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An Examination of the Pharmacology of Spermidine and Its Possible Role in the Central Nervous System

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AN EXAMINATION OF THE PHARMACOLOGY OF SPERMIDINE AND ITS POSSIBLE ROLE IN THE CENTRAL NERVOUS SYSTEM

by

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VITA

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PURPOSE

The purpose of this investigation will be to study the pharmacology of the polyamine spermidine in further detail. The general pharmacology, including acute toxicity, *in vitro* bioassay and *in vivo* physiologic responses will be described. The variability of the toxicity of spermidine during a programmed light-dark cycle will be studied. A possible interaction between spermidine and histamine will be studied in relation to the daily fluctuation of their levels in the central nervous system as well as their effect on pentobarbital-induced sleeping time.

INTRODUCTION

HISTORY,DEFINITION AND CHEMISTRY

The polyamines are a diverse group of nitrogenous bases widely distributed throughout nature. They include aliphatic , heterocyclic and aromatic derivatives but most recent interest has centered on a series of aliphatic di- and polyamines. The structures of the major members of this group are seen in Table I .

Antoni Van Leeuwenhoek is credited with the recognition of crystals of spermine in samples of human semen (Leeuwenhoek , A. Van,1678 ; Smith,T.A. , 1972). It was not until well over two hundred years later however that the correct structure of spermine was established and its synthesis accomplished (Dudley,H.W. et al., 1926).

THE MAJOR POLYAMINES

$NH_2(CH_2)_3NH_2$	1,3- diaminopropane
$NH_2(CH_2)_4NH_2$	1,4- diaminobutane (putrescine)
$NH_2(CH_2)_5NH_2$	1,5- diaminopentane (cadaverine)
$NH_2(CH_2)_3NH(CH_2)_4NH_2$	spermidine
$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	spermine

TABLE I

OCCURRENCE IN BIOLOGIAL MATERIAL

PLANTS

There has been relatively little interest in the presence and possible role of polyamines in plant material (Smith, T.A., 1972). Putrescine is the major member of the series found in most plant species (Smith,T.A., 1970). Spermidine and spermine are also present in most higher plants in quantities ranging from 0.01 - 0.25 micromoles per gram (Smith, T.A., 1970). These compounds are predominantly found in the leaves of these plants and in the embryo of the seed, particularly during earlier stages of germination.

BACTERIA AND VIRUSES

Polyamines are found in relatively high concentration in T-even bacteriophages of E. coli (Ames et al., 1958). They are present in concentrations which can neutralize up to forty percent of the DNA phosphorus of the virus. Unlike many strains of bacteria, the bacteriophage does not take up polyamines from the medium and thus their presence in these organisms represents the best evidence for the in vivo association of the polyamines and nucleic acids.

Spermidine and trace quantities of spermine have been identified as constituents of Turnip Yellow Mosaic virus (TYMV). The total amount found would be sufficient to neutralize approximately twenty percent of the viral RNA (Beer, S.V. and Kosuge, T., 1970).

Polyamines are found in a variety of bacteria, yeasts and fungi (Tabor, H. and Tabor, C.W., 1972). Their concentration in bacteria is in part dependent on the characteristics of the medium used and the conditions of growth present. Higher concentrations are found when organisms are grown in a medium enriched with polyamines. The mechanisms of uptake of polyamines by E. coli have been studied by Tabor and Tabor (1966). They described (a) a non-energy dependent adsorption of amines onto the cell surface and (b) an energy-dependent uptake, stimulated by glucose, into the interior of the cell. Higher concentrations of polyamines are also found during periods of growth of bacterial cultures. Gram-negative bacteria contain large amounts of polyamines while gram-positives contain relatively little or none at all (Herbst et al., 1958).

Significant quantities of polyamines are found in the fungus Neurospora crassa. A magnesium deficient medium can stimulate polyamine synthesis in this orgnaism (Viotti, A., et al., 1971).

ANIMALS

The highest concentrations of spermidine and spermine in animal tissues are found in pancreas, prostate and human semen (Rosenthal, S.M. and Tabor, C.W., 1956; Tabor, H. and Tabor, C.W., 1964). The high concentration of spermine in human semen forms the basis for some medicolegal studies. Table II summarizes the reported levels of spermidine and spermine in selected animal tissues.

Pfeiffer et al. (1970) and Iliev et al. (1968) have studied polyamine levels in whole blood of humans. Levels of spermidine were detectable in the range of 0.9 to 1.0mg./ml. and the concentration of spermine was 1.38 to 1.48 mg./ml. They found no correlation with the age or sex of the subjects.

The molar ratio of spermidine to spermine varies with the tissue and its stage of development (see below) but in general the ratio is between 1.0 and 2.0 except in the case of testes, kidney, skeletal muscle and human semen and prostate, where the ratio is less than one in most species. Janne et al. (1964) have shown in the rat that levels of spermidine decrease significantly after birth in all

THE CONCENTRATION OF POLYAMINES IN SELECTED ANIMAL TISSUES

ORGAN	ANIMAL SOURCE	SPERMIDINE (ug/gm)	SPERMINE (ug/gm)
Semen	Human	trace	3300
Prostate	Human		495
	Rat	1122	1155
Liver	Human		495
	Rat	230	235
	Mouse	235	155
Spleen	Human		45
	Rat	230	134
Bone Marrow	Human		148
Pancreas	Rat	1250	194
	Mouse	410	202
	Guinea pig	312	280
Testis	Rat	55	105

TABLE II

tissues examined while spermine shows relatively little change except in the case of brain and skeletal muscle. Thus, the molar ratio of the two decreases over the first few months of life.

By radioautography, Dion and Herbst (1967) have shown a localization of spermidine within the nucleus of the salivary gland of Drosophila melanogaster. Application of this technique to the intracellular localization of polyamines in other systems has not been successful.

The presence of spermidine and spermine in neural tissue was unappreciated until Kremzer (1966) and Michaelson (1967) identified them as contaminants in the existing method for the detection of histamine. That method, described by Shore, Burkhalter and Cohn in 1959, involved the n-butanol extraction of alkalinized perchloric acid tissue extracts which were then condensed with o-pthaldialdehyde (OPT) to form a fluorescent product. (Histamine and spermidine have very similar fluorescence spectra when complexed with OPT. Both are maximally activated at 350 mu.) Histamine is read at 450 mu and spermidine at 400 mu. Using this method Michaelson and Dowe (1963) had reported histamine levels of 2.2 nanomoles per gram of guinea pig brain. Carlini and Green (1963) reported similar levels of 2.2 nanomoles per gram using the same method but found only 0.48 nanomoles per gram

when a biological assay (guinea pig ileum) was used. In a study of the diurnal variability of histamine levels in rat caudate nucleus, Friedman and Walker (1968) reported histamine levels ranging from 10.5 to 20.1 nanomoles per gram. Eliminating spermidine and spermine using the method of Anton and Sayre (1968) the true levels of histamine were subsequently found to vary between 0.63 and 1.08 nanomoles per gram (Friedman, A.H. and Walker, C.A., 1969). Thus, spermidine and spermine were present in the rat caudate nucleus in a concentration twenty times that of histamine. The method of Kremzer has subsequently been modified to separate histamine, spermidine and spermine (Medina, M. and Short, P.A., 1966; Iliev, V. et al., 1968; Hakanson, R., and Roonberg, A., 1973). Michaelson has found the phosphorylated cellulose resin used in the latter methods unreliable and has subsequently described the use of another phosphonic acid cation exchange resin, Bio-Rex-63 (Bio-Rad Laboratories, Richmond, California) which is more stable and consistent (Michaelson, I.A. and Coffman, P.A., (1969). It is the method of Michaelson and Coffman that has been modified for use in this study. A highly sensitive and specific enzymatic isotopic method for histamine has subsequently been devised (Snyder, S.H., et al., 1966; Taylor, K.M. and Snyder, S.H., 1971; Taylor, K.M. and Snyder, S.H., 1972). Reference will be made to results obtained with this method. More recent methods for the detection of

polyamines have been described utilizing alternate cation exchange resins (Marton et al.,1973; Tabor,H. and Tabor,C.W.,1973) or gas chromatography (Denton,M.D. et al.,1973). Recently a method has been developed for the detection of putrescine (Harik,S.I. et al., 1973).

Significant levels of spermidine and spermine are present in both the central and peripheral nervous systems. Reported concentrations have been variable, in part a function of the methodology employed but perhaps also,as will be shown later in this study, a function of the point in the light-dark cycle at which the tissues were taken. Tables III and IV summarize levels reported in whole brain homogenates of several species.

Despite their structural similarity and metabolic interrelationships, the regional distribution of spermidine and spermine are not similar. Shaw and Pateman (1973) studied the levels of these amines in selected brain areas of multiple species. In a thorough analysis of sheep brain, highest levels of spermidine were detected in regions rich in myelinated fiber tracts such as subcortical white matter (115 ug/gm), cerebellar white matter (102ug/gm), pons (112ug/gm) and optic chiasm (118 ug/gm). Significantly lower levels were found in neuronal masses such as cortical grey (25ug/gm), caudate nucleus (24 ug/gm) and hypothalamus (37 ug/gm). Although absolute values

REPORTED LEVELS OF SPERMIDINE IN WHOLE BRAIN OF VARIOUS SPECIES

ANIMAL	WHOLE BRAIN SPERMIDINE (ug/gm)	REFERENCE
Guinea pig	35.2	Kremzer (1966)
Rabbit	45-48	Kremzer (1966)
	62-71	Shimizu et al. (1964)
Mouse	65	Seiler (1973)
	40	Shimizu et al. (1965)
	45	Giorgi et al. (1972)
Rat	98	Rosenthal and Tabor (1956)
	44	Kremzer (1970)
	70	Pearce and Schanberg (1969)
Fish	62	Seiler and Lamberty (1973)

TABLE III

REPORTED LEVELS OF SPERMINE IN WHOLE BRAIN

OF VARIOUS SPECIES

ANIMAL	WHOLE BRAIN SPERMINE (ug/gm)	REFERENCE
Rabbit	29	Shimizu et al. (1964)
Mouse	41	Giorgi et al. (1972)
Rat	41	Tabor and Tabor (1964)
Fish	19	Seiler and Lamberty (1973)

TABLE IV

varied somewhat, essentially similar distributions were found in rat, rabbit, dog and human specimens. Cervical cord was studied in the rat and rabbit and contained higher concentrations of spermidine than any other area assayed. Studies of rat brain by Shimizu (1964) showed a similar distribution and included analysis of peripheral (sciatic) nerve which contained relatively low levels compared to areas rich in central rather than peripheral myelin. Kremzer (1970) and Kremzer et al.(1970) also analysed human brain with results similar to those of Shaw and Pateman. In addition , no significant difference was found in the spermidine concentration of different cortical areas. (Cortical white matter contains considerably more spermidine, 92-122ug/gm , than adjacent cortical ribbon, 20-38ug/gm.(Shaw and Pateman,1973)). The concentration of spermidine in rat caudate nucleus was found to be 60ug/gm by Shimizu et al.(1964) while the concentration of authentic histamine in this structure is 0.05-0.15 ug/gm (Friedman and Walker ,1969), explaining the spuriously high levels of histamine reported when spermidine was not separated prior to condensation with OPT.

Shaw and Pateman (1973) found less remarkable and less consistent regional variability of spermine levels. In sheep brain , highest levels were found in the pituitary,trigeminal nerve base, optic chiasm, lateral geniculate bodies and the cerebral peduncles. Examination of two human brains showed inconsistent levels but there was a

tendency toward high levels in more caudal brain areas, i.e. areas of the brainstem. In rabbit brain Shimizu et al. (1964) found highest levels of spermine in cortex and caudate nucleus (36ug/gm) with a progressive decline in more caudal regions such as lumbar spinal cord (9ug/gm) and sciatic nerve (5ug/gm). Kremzer et al. (1970) did not find any significant difference in the spermine concentration in several areas of sheep brain.

Neither Shaw and Pateman (1973) nor Kremzer et al. (1970) could find detectable levels of spermidine or spermine in human cerebrospinal fluid.

The absence of a reliable method for the determination of putrescine has limited studies of this compound. Employing an enzymatic-isotopic assay found to be both sensitive and specific, Harik et al. (1973) have detected significant levels of putrescine in mouse brain (0.17ug/gm) and rat brain (0.17ug/gm).

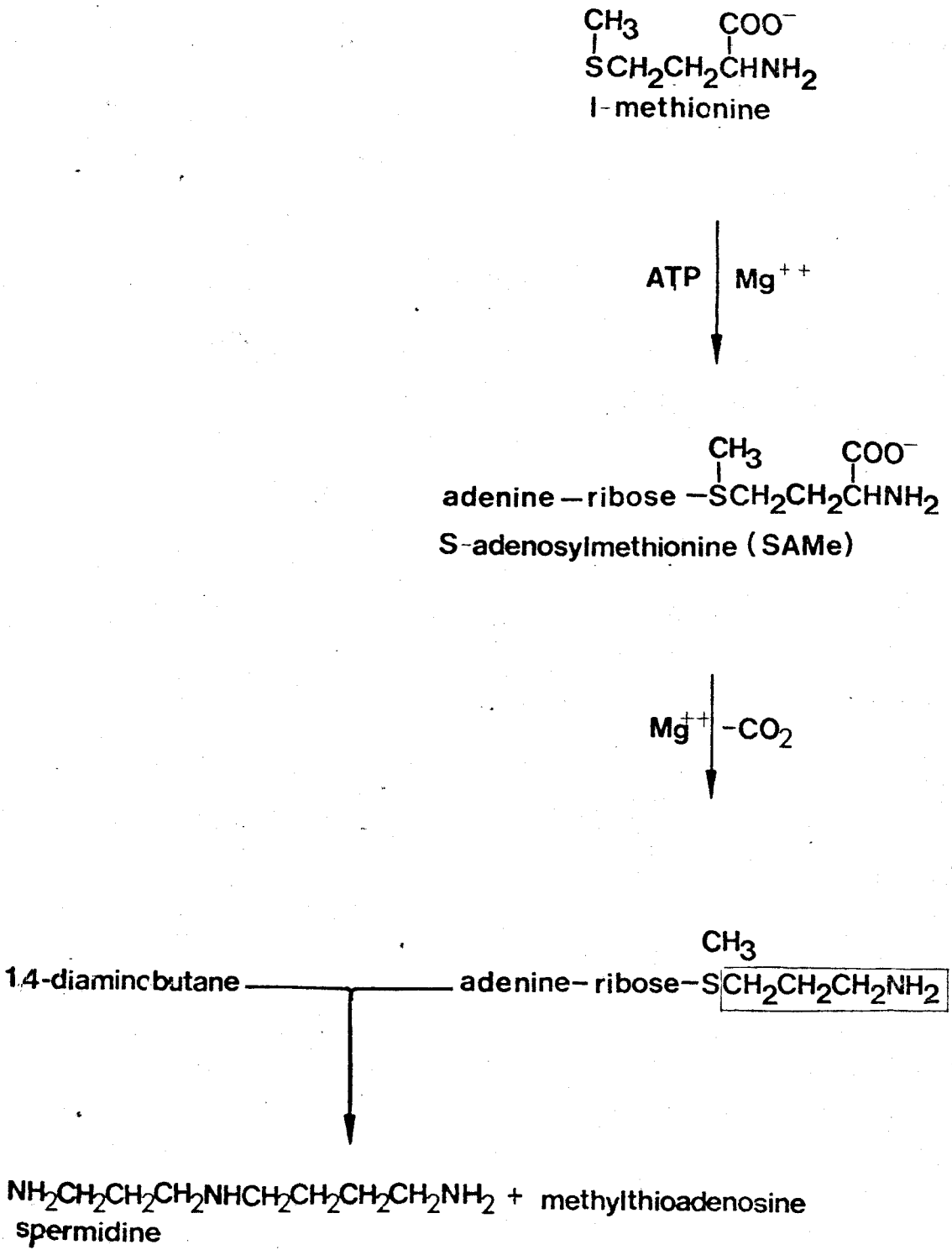
METABOLISM

SYNTHESIS

The biosynthesis of putrescine , spermidine and spermine has been well characterized in microorganisms, particularly E. coli (Bachrach, U., 1970; Tabor, H. and Tabor, C.W., 1972). Studies of animal tissue have included chick embryo (Raina, A., 1963), rat liver, especially during regeneration (Janne, J., 1967; Slimes, M., 1967; Russell, D.H. and Snyder, S.H., 1969; Snyder, S.H. and Russell, D.H., 1970; Russell, D.H. and McVicker, T.A., 1971) and rat prostate (Pegg, A.E., 1970; Pegg, A.E. and Williams-Ashman, H.G., 1970). A review of preliminary studies of higher plants has recently been presented by Smith (1970).

In all organisms studied the formation of spermidine involves the addition of a propylamine group to 1,4-diaminobutane as seen in Figure 1. S-adenosylmethionine (SAMe) is perhaps most important in biological systems as an active methyldonor (Baldessarini, R.J., and Kopin, I.J., 1966). Enzymes for its formation from ATP and methionine

FIGURE 1. BIOSYNTHESIS OF SPERMIDINE



are present in most organisms. SAMe is decarboxylated enzymatically as seen in the figure. In mammalian systems this enzyme is markedly dependent on the presence of 1,4-diaminobutane (Pegg, A.E., and Williams-Ashman, H.G., 1968). Mg^{++} is required for the enzyme found in E.coli. A pyridoxal phosphate inhibitor, NSD-1055 inhibits the mammalian enzyme but not the E. coli enzyme. Recently, methylgloxal Bis (guanylhydrazine) has been shown to be a specific competitive inhibitor of SAMe decarboxylase (Williams-Ashman, H.G. and Schenone, A., 1972; Pegg, A.E., 1973).

Propylamine transferase catalyses the last step in the formation of spermidine. The purified enzyme has no known cofactors. Negative feedback is exerted on its activity by the products of the reaction. Although in some systems spermidine may act as a substrate of the same enzyme (resulting in the formation of spermine), this is not the case in mammalian systems (Janne, J. et al., 1971). A second enzyme for the formation of spermine from SAMe and spermidine has been purified from both rat brain and regenerating rat liver (Hannonen, P. et al. 1971; Hannonen, et al., (1972).

In the central nervous system, SAMe-Decarboxylase activity is highest in the cerebral cortex and cerebellum (Snyder et al., 1973). After intraventricular injection of labelled putrescine, these investigators

found that virtually all of the putrescine had been converted to spermidine and spermine by 48 hours. Like tracer doses of other amines such as tyrosine and histidine, 60 to 70% of the dose was converted within the first twenty minutes. Overall, spermine accounted for only 10 to 25% of the activity detected. The tracer was initially distributed to midbrain, hypothalamus and cerebellum but levels declined sharply in these regions. Spermidine activity was detected as early as twenty minutes and reached peak levels in medulla-pons, internal capsule and corpus striatum by 48 hours. Activity in spermine could not be detected until 48 hours and peak levels did not occur until the fifteenth to nineteenth days, particularly in cerebral cortex and cerebellum.

Kremzer et al. (1970) studied the regional distribution of propylamine transferase activity in several regions of sheep brain. Like SAMe decarboxylase activity and in contrast to the distribution of ornithine decarboxylase, highest levels were found in the cerebral cortex.

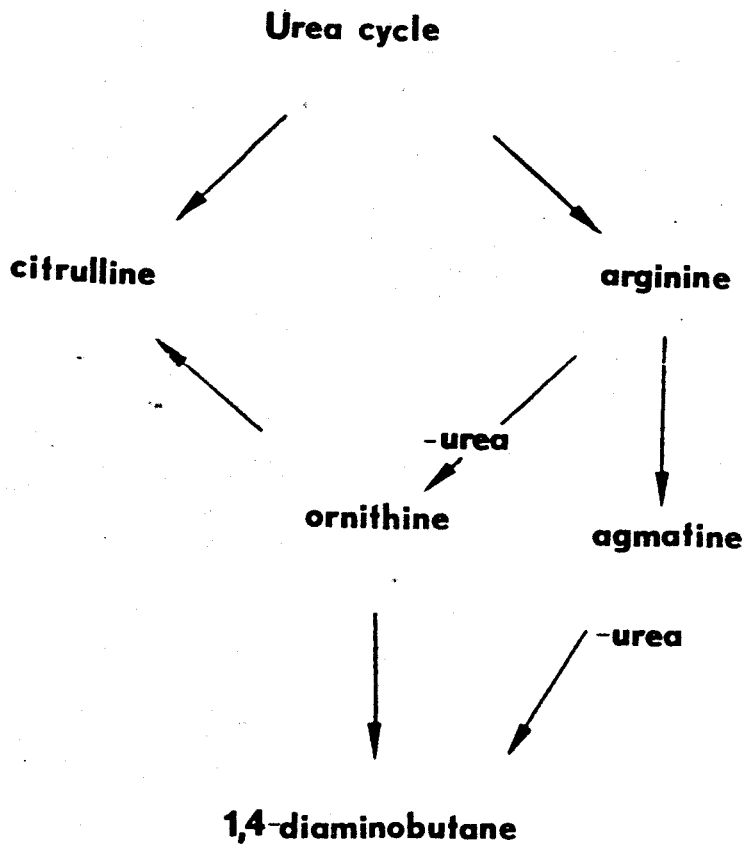
In animal tissue, ornithine is the only source of putrescine for polyamine synthesis. The conversion is catalysed by ornithine decarboxylase, a specific enzyme found in most tissues. It has been purified from rat prostate (Janne, J. and Williams-Ashman, H.G., 1970) and regenerating rat liver (Friedman, S.J., et al, 1972) and found to

require pyridoxal phosphate as a cofactor. Its activity is stimulated by its substrate but also by several hormones including testosterone (rat prostate), hydrocortisone, growth hormone, insulin, glucagon and thyroxin (rat liver) and luteinizing hormone (rat ovary)(Russell, et al., 1970; Pegg, A.E., et al, 1970; Panko, W.B. and Kenney, F.T., 1971; Kobayaski, Y. et al, 1971). Harik et al. (1974) have shown alpha-hydrazino-ornithine to be a specific competitive inhibitor of the enzyme. Hayaski et al. (1972) have shown some diurnal variability in the activity or ornithine decarboxylase which they related to protein intake, but lighting conditions were not controlled in their study. Hernandez et al. (1973) have shown variability or ornithine decarboxylase during the estrous cycle of rats, peak levels occurring during the proestrous phase.

Both plants and bacteria can form 1,4-diaminobutane by an alternate pathway from arginine. Agmatine is formed by decarboxylation of arginine, catalysed by a specific enzyme not found in animals. Putrescine may be formed from agmatine with the production of urea. At least in the case of E.coli, ornithine decarboxylase accounts for the synthesis of 75-98% of the putrescine (Morris et al., 1970). Plants, on the other hand, depend on arginine decarboxylase.

Figure 2 summarizes the pathways of putrescine synthesis.

FIGURE 2. BIOSYNTHESIS OF 1,4-DIAMINOBUTANE



CATABOLISM

Diamine oxidase is a copper-containing enzyme purified from hog kidney. It catalyses the oxidation of short chain diamines, as well as histamine, to the corresponding aldehyde. Gamma-aminobutyraldehyde, the product of putrescine oxidation, cyclizes to delta^1-pyrroline or it may be further oxidized to gamma-aminobutyric acid (GABA) (Razin, S. et al., 1959). The functional significance of this pathway for the formation of the putative neurotransmitter GABA is unknown.

Several bacterial species are capable of converting spermine to spermidine and beta-alanine. Spermidine may then be further cleaved to form another molecule of beta-alanine and GABA (Razin, S. et al., 1959).

Kakimoto et al. (1969) reported the presence of putreanine (N-(4-aminobutyl)-3-aminopropionic acid) in the brain of rabbits and rats. They felt this compound was unique to the nervous system since it was not detected elsewhere. Nakajima (1973) has subsequently detected trace levels in rat liver and has demonstrated that it is a product of spermidine catabolism. The significance of its relative localization to central nervous tissue is unclear.

PUTATIVE FUNCTIONS OF THE POLYAMINES

INTERACTIONS WITH NUCLEIC ACIDS

Low concentrations of polyamines have a remarkable stabilizing effect on DNA. The melting temperature of DNA is markedly increased in the presence of concentrations of spermine and spermidine as low as 10^{-5}M (Tabor, H., 1962; Stevens, L., 1970). The two more likely ways this is accomplished are that either the amines neutralize the phosphate groups of the DNA (reducing the mutual repulsion between the strands) or that they form bridges between the strands. X-ray diffraction and electron microscopic studies have favored the latter possibility (Chevaillier, P., 1969; Sutvalsky,M. et al., 1969). The polyamines do not alter the conformation of the DNA helix but, when interacting with RNA, this polynucleotide becomes folded and more compact.

Polyamines promote the association of ribosomal subunits to form 70S or 80S particles. Furthermore, they promote the attachment of transfer -RNA and messenger -RNA to ribosomes. Finally, these compounds have been found in vitro to stimulate the activity of DNA-primed RNA polymerase and possibly also DNA-primed DNA polymerase. Datta et al.(1969)

and Giorgi (1970) have confirmed some of these observations in preparations of rat cerebral cortex.

In vivo studies of the association of nucleic acids and polyamines have not been accomplished. Because of their polycationic nature, the polyamines redistribute because of their tendency to associate with subcellular fractionation (Michaelson, I.A. and Smithson, H.R., 1971).

GROWTH AND DEVELOPMENT

In studies of chick embryo, Raina (1963) demonstrated a marked acceleration of de novo spermine and spermidine synthesis reaching peak values on the sixteenth day of incubation followed by a sharp decrease. A second increase in the concentration of these polyamines occurred between the tenth to fourteenth day after hatching, again followed by a sharp decline to adult levels. Similar results were obtained by Calderera et al. (1965) who demonstrated a close association with nucleic acid levels. Manen and Russell (1973) have shown a remarkable cyclic increase in ornithine decarboxylase and propylamine transferase activity in the early stages of sea-urchin embryogenesis. Enzyme activities increased soon after fertilization, reaching peak levels within thirty minutes. Enzyme activities and polyamine levels decreased just prior to each subsequent cleavage. Herbst and Dion (1970) have shown similar correlations in Drosophila.

Specifically in brain, Calderera et al (1969) have found peak polyamine levels twelve to fourteen days after fertilization. Levels then gradually decline but, as in whole embryo and in other isolated organs, a second peak occurs ten to fourteen days after birth, declining thereafter to adult levels. Parallel changes are seen in nucleic acid levels. Pearce and Schanberg (1969) have made the interesting observation that histamine levels undergo similar changes but precede those of spermidine by 24 to 48 hours.

An association between polyamines and accelerated tissue growth is apparent in several different models. In regenerating rat liver, ornithine decarboxylase activity triples one hour after partial hepatectomy (Russell, D.H. and Snyder, S.H., 1969a). By four hours levels are ten times basal levels. Parallel changes occur in RNA levels while increases in DNA do not occur until 16 or 18 hours later. Administration of actinomycin D or cycloheximide prior to or up to thirty minutes after the operation prevents the increase in enzyme activity. Using these drugs, the half-life of newly synthesized ornithine decarboxylase was found to be 11 minutes , **the** shortest known for mammalian enzymes (Russell, D.H. and Snyder, S.H., 1969b; Snyder, S.H. and Russell, D.H., 1970). Hypophysectomy also prevents much of the increase in ornithine decarboxylase and treatment with growth hormone reverses this effect (Russell, D.H. and Snyder, S.H., 1969c).

CENTRAL NERVOUS SYSTEM

In the nervous system, Kremzer (1970) has found increased conversion of putrescine to spermidine in peripheral nerve adjacent to a point of transection, presumably due to activation of regenerative processes. There have been several reports of elevated levels of polyamines and their associated enzymes in neoplastic tissue including tumors of the CNS (Kremzer et al., 1970; Snyder, S.H. and Russell, D.H., 1969b; Ruseell, D.H., 1973). Levels of urinary polyamines may be elevated in patients harboring neoplasms (Russell, D.H., et al, 1971; Russell, D.H., 1973). Overall however, despite the high concentrations, there is little evidence concerning the function of polyamines in the CNS.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Only male Swiss Albino mice weighing between twenty and thirty grams were used in the present studies and were obtained from the same commercial source (Abrams Scientific Animal Farm, Madison, Wisconsin). They were housed four per cage in standard polyethylene cages (30 x 18 x 12 cm.) with commercial pine sawdust as bedding. Unless otherwise indicated, food (Purina Rat Chow) and tap water were provided ad libitum.

PROGRAMMED LIGHT-DARK CYCLE

All animals were housed in quarters with an automatically-timed daily cycle of twelve hours of light and twelve hours of darkness. Light was provided using commercial Vita-Lite fixtures, emitting a spectrum of wavelengths similar to that of natural sunlight (Wurtman and Weisel, 1969; McGuire et al., 1973). The ambient temperature in the quarters did not vary more than two to three degrees throughout the cycle.

Extraneous noise was kept to a minimum and the quarters were entered as seldom as possible during periods of entrainment and experimentation.

All animals were adapted to the above conditions for at least three weeks prior to experimentation. This has been shown to be more than adequate for the entrainment of most known endogenous rhythms in rodents (Bruce, 1960; DeCoursey, 1960).

To facilitate various activities in the animal quarters during the dark phase of the cycle a small lamp emitting a dim red light was used since this spectrum has the least effect on circadian rhythms (McGuire et al., 1973).

REAGENTS

1. Only distilled-deionized water was used for preparation of solutions.
2. Spermidine phosphate was obtained from Sigma Chemical Co. (St. Louis, Mo.). Solutions for animal injection and for bioassay experiments were prepared in 0.9% sodium chloride.
3. Cyproheptadine hydrochloride was kindly supplied by the Merck, Sharp & Dohme Company (West Point, Pa.). Because of its low solubility in water, fine emulsions were made in 5% methylcellulose. Injections were made intraperitoneally with an 18 gauge needle at 0.1 ml./kg.

4. Tripelenamine hydrochloride was kindly supplied by CIBA (Summit, N.J.). Solutions were prepared in 0.9% sodium chloride. All doses were injected in a volume of 0.1 ml./kg.
5. Pentobarbital sodium was prepared in 0.9% saline. A fresh solution was prepared prior to each individual experiment.
6. Acetycholine hydrochloride was purchased from the Sigma Chemical Co. (St. Louis, Mo.). Solutions for bioassay were freshly prepared in Tyrode's solution.
7. Decaborane ($B_{10}H_{14}$) was obtained from Alfa Inorganics, Inc. (Beverly, Mass.). The powder was dissolved in corn oil for intraperitoneal injection.
8. Bio-Rex-63 was purchased from the Bio-Rad Company (Richmond, California).
9. O-phthaldialdehyde (OPT) was obtained from Sigma Chemical Company (St. Louis, Mo.).
10. Tyrode's solution was prepared according to the following formula.

REAGENT	CONCENTRATIONS IN GM./LITER
1. NaCl	8.00
2. KCl	0.20
3. $CaCl_2$	0.20
4. $NaHCO_3$	1.00
5. $MgCl_2 \cdot 6H_2O$	0.10
6. $NaH_2PO_4 \cdot H_2O$	0.05
7. Glucose	1.00

The solution was prepared fresh for each separate preparation.

TOXICITY STUDIES

Intravenous injections of spermidine were given by tail vein. Intraperitoneal injections of spermidine and other preparations were given in the usual fashion. All injections were given in a volume of 0.1 ml./kg. After the injections, animals were replaced in their original cages for an observation period of thirty minutes in the case of the intravenous toxicity study or for 270 minutes in the case of the intraperitoneal study. Appropriate dose levels were used in order to calculate the LD50. Identical studies were carried out at regular intervals (four hours) throughout the light-dark cycle to determine the diurnal variation in the LD50. Mortality was attributed to the

time period when the dose was given. Calculation of the LD50 was given. Calculation of the LD50 was made according to the method of Litchfield and Wilcoxen (1949). Post-mortem examination was performed immediately after death in several representative animals and some simple observations recorded.

PROTECTION FROM THE TOXICITY OF SPERMIDINE

Animals were pretreated with either 0.9% saline, cyproheptadine or tripelenamine given intraperitoneally thirty minutes prior to the administration of toxic doses of spermidine. As in previous studies, a 270 minute observation period was used in the determination of total mortality. In separate experiments, increasing doses of cyproheptadine and tripelenamine were employed and a dose response curve constructed.

MOTOR ACTIVITY RHYTHM

Four animals had been entrained in the programmed light-dark cycle for three weeks as described above. Each was then adapted for three days to a cage equipped with a photo-cell. One count was recorded each time the animal interrupted the photobeam. Food and water were given ad lib. The pellets of rat chow and water container were located at the greatest distance from the photobeam as possible so as to minimize the recording of simple feeding activity. On the

fourth day the counts were recorded at regular intervals throughout the light-dark cycle. The average number of counts per hour for all four animals was then plotted versus time of day. Correlations were made with the diurnal variation of several different parameters of interest in this study such as the LD50.

IN VITRO ACTIVITY

Male guinea pigs starved for the preceding 24 hours were killed at 1000 hours by a blow on the head. The abdomen was opened and the distal half of the small intestine to its junction with the cecum was isolated. The mesentery was carefully stripped away with fine ophthalmic scissors. The lumen was carefully cleared of fecal material by gently passing Tyrode's solution at 30^{o} C through it using a standard blunt-tipped pipette. During preparation the strip of intestine was maintained in oxygenated Tyrode's solution. The distal 3 cm. segment was discarded. A 2-3 cm. segment was then anchored at one end to a 10 milliliter bath while the other end was connected to a strain gauge. No counter balancing weight was used. Bath temperature was maintained at 30^{o}C with a continuous stream of fine bubbles of oxygen. All solutions for injections were maintained in vials cooled with ice.

A three minute agonist injection cycle was maintained. The bath was washed thirty seconds after injection of the agonist. All solutions

injected had been prepared in Tyrode's solution and thus their pH was identical to that of the perfusion bath. After successive responses to the agonist did not vary by more than two to three millimeters in amplitude, the effect of pretreatment with spermidine prior to the agonist was evaluated. The result was expressed as percent reduction in the amplitude of the average control response to the agonist. Injections of agonist alone were subsequently continued until the response returned to pretreatment levels. The effect of an isolated dose of spermidine on the spontaneous activity of the preparation was also determined.

IN VIVO ACTIVITY

Healthy mongrel dogs were anesthetized with intravenous pentothal sodium. Tracheostomy was performed on all animals. A continuous infusion of 5% dextrose in physiologic saline was maintained. Respirations were recorded via a string attached to the chest wall and to a strain gauge. Electrocardiogram was monitored with standard limb leads. Blood pressure was monitored via an indwelling catheter in the femoral artery connected to a pressure transducer. All injections were made through an indwelling femoral venous catheter. Responses were recorded on a standard Grass^{R} polygraph.

SLEEPING TIME IN MICE

Sleep was induced with intraperitoneal doses of sodium pentobarbital. Onset of sleep was defined as the moment of complete loss of righting reflex. Cessation of sleep was marked when the animal was able to walk 6 inches in any direction (without prodding). The variation in response to a single dose of pentobarbital was determinted at regular intervals (four hours) throughout the light-dark cycle. Rectal temperature was monitored during several experiments using a soft-tipped thermister probe connected to a continuous temperature monitor (Yellow Springs Instrument Co.).

To determine the effect of injections of spermidine on pentobarbital-induced sleep, groups of animals were pretreated with various doses of either spermidine or 0.9% saline. (In addition, a group of animals was given no pretreatment in order to determine the effect of the act of intraperitoneal injection alone on subsequent sleeping time.) Injections of pentobarbital sodium were given at several intervals after pretreatment. The optimum pretreatment interval was determined and used in all subsequent experiments. Although pretreatment with physiologic saline did not differ significantly from no pretreatment at all, this procedure was continued as the control in subsequent studies.

Identical experiments were carried out at four hour intervals during the light-dark cycle to determine the diurnal fluctuation in the alteration in pentobarbital-induced sleeping time produced by spermidine.

Central levels of histamine were depleted by the use of intraperitoneal injections of decaborane as described by Medina et al. (1969). (This effect was confirmed by fluorimetric assay as described below.). Sleep was induced with pentobarbital sodium twenty-four hours after the injection of decaborane and in a group of controls not pretreated with decaborane. Some animals were pretreated with both decaborane and spermidine, 24 hours and 30 minutes respectively, prior to the injection of pentobarbital. Control animals received injections of corn oil (for decaborane) or 0.9% saline. All animals (including controls) were fasted after injection of the decaborane. Sleeping time was then determined as described above.

Central histamine and spermidine levels were determined at 15 minute intervals after the induction of sleep with pentobarbital. Two control animals were sacrificed at time zero. Either pentobarbital or 0.9% saline was then injected into separate groups of animals. Two animals were sacrificed from the treated group at 15, 30, 45 & 60 minutes after injection. At 60 minutes two control animals were again sacrificed along with the two treated animals.

FLUORIMETRIC ASSAY OF SPERMIDINE AND HISTAMINE

Tissue levels of histamine and spermidine were determined according to the method of Michaelson and Coffman (1969) with minor modifications described below. The reliability and sensitivity of the method

were evaluated. Aliquots of standard solutions of both amines were carried through the entire procedure both alone and in combination with aliquots of tissue.

Animals were taken from the quarters and immediately killed by decapitation without anesthesia. The brain was carefully removed to the cervicomedullary junction. The pons and medulla were separated as a single specimen. The remainder of the brain (mesencephalon, thalamus-hypothal. and hemispheres) was taken separately. Specimens were then placed in an aluminum-foil envelope, which was immersed in liquid nitrogen. This was easily accomplished within one to two minutes after decapitation. If needed, the entire liver was then removed and treated in a similar fashion. All samples were kept frozen until assayed. All tissues were assayed within one week of removal.

PREPARATION OF TISSUES FOR ASSAY

1. Tissues for assay were homogenized in 0.4N Perchloric acid containing 1 mg./ml. of EDTA. The volume of perchloric acid was equal to four times the weight of the tissue specimen in grams.
2. The homogenizing vessel was kept in a container of ice during the homogenization.
3. Homogenates were maintained in an ice bucket for ten minutes before centrifugation.

4. The homogenates were then centrifuged (in their homogenizing vessel) at approximately 2200 rpm for ten minutes. For this short interval of spinning the tubes were cool at the termination of centrifugation.
5. A four ml. aliquot of the supernatant was then transferred to a shaker tube containing 0.5 ml. of 5N NaOH, 1.5 gm. of solid NaCl, 12 ml. of n-butanol.
6. Tubes were then shaken four times in a vortex mixer for thirty seconds, resulting in excellent mixing.
7. Shaker tubes were then centrifuged at 1000 rpm for ten minutes.
8. The aqueous phase was then aspirated. Some solid NaCl was usually left in the bottom of the tube after this procedure.
9. The remaining butanol phase was then shaken as above with 5 ml. of a salt-saturated solution of 0.1N NaOH.
10. Step #7 was repeated.
11. A 10 ml. aliquot of the butanol phase was then transferred to another shaker tube containing 20 mg. of n-heptane and 1.5 mg. of 0.1N HCl.
12. The tubes were shaken on the vortex mixer as above.

13. Step #7 was repeated.
14. One ml. of the aqueous phase was diluted in 9 ml. of 0.2M phosphate buffer and adjusted to pH 6.0-6.2 with 1N NaOH. (3-4 drops from a pasteur pipette were usually required)
15. The entire solution was then applied to the column.

PREPARATION OF THE RESIN

1. An adequate volume of resin was hydrated in 4 volumes of distilled-deionized water.
2. The mixture was then heated to 80 degrees (Fahrenheit) in a water bath for thirty minutes with constant stirring.
3. The resin was then allowed to settle and the water plus any slowly settling particles of resin were decanted.
4. The resin was then washed with 2 volumes of pure ethanol for ten minutes while stirring constantly.
5. The ethanol was then removed through a Buchner funnel with tap suction.
6. The resin was rehydrated in 4 volumes of distilled-deionized water while stirring constantly for 5 minutes.
7. The water was decanted along with any slowly settling particles.
8. Resin was then slurried in a convenient volume of 2N HCl. One-half the volume of the resin bed was more than adequate.

9. The column was formed by transferring the slurry via pipette to the bed. A 4 cm. bed of resin was appropriate for use on a column of 5 millimeter diameter.

ELUTION OF THE RESIN

1. 10 ml. of 2 N HCl were passed through the column. This was sufficient to make a strongly acidic eluent.
2. A sufficient quantity of deionized water was then passed through the column until the eluent was no longer acidic. Thirty ml. of water was usually more than enough. The pH of the eluent was followed with long then short range pH paper.
3. A sufficient quantity of 1.5M NaOH was then passed through the column to make the eluent alkaline. One-half ml. of the 1.5 M NaOH diluted in 10 ml. of deionized water was adequate.
4. Deionized water was again used to remove all traces of alkali. Twenty ml. were usually required.
5. 1 ml. 2N HCl diluted in 10 ml. deionized water was applied to make the resin eluent acidic once more.
6. Deionized water, usually 20 ml., was used to elute all traces of the acid.

7. 20-30 ml. of 0.2M buffer were then passed through the column. Amines or samples were added at this time by diluting them in the final 10 ml. aliquot of buffer being added.

ELUTION OF AMINES

1. The resin was first eluted with 50 ml. of .02N HNO_3. The eluate was discarded.
2. 50 ml. 0.1N HNO_3 was used to elute the histamine. This is collected, 1 ml. per tube, in test tubes suitable for development and reading.
3. 40 ml. of 0.2N HNO_3 then eluted the spermidine and this was collected in 4 aliquots of 10 ml. each.

DEVELOPMENT

1. 1 ml. of each standard solution were developed along with a 1 ml. reagent blank consisting of the eluting agent used for whichever amine was being developed.
2. Tissue blanks were prepared by adding the phosphoric acid prior to mixture with OPT. The final 1 ml. aliquot from both the histamine and spermidine eluates were used for this purpose.

3. Formation of the histamine - OPT fluorophore.
   a. 0.1 ml. of 4N NaOH 0 mix well.
   b. 0.05 ml. of 0.5% OPT - mix well and after 2 minutes add
   c. 0.2 ml. of 3.5 M H_3PO_4
4. Formation of the spermidine fluorophore.
   a. 0.3 ml. 1N NaOH
   b. 0.05 ml. 0.2% OPT - mix well and after 6.5 minutes add
   c. 0.15 ml. 2.5M H_3PO_4

FLUORIMETRIC READING

1. Slit widths in order of sequence from light source to reading chamber in the Aminco-Bowman Spectro-fluorimeter were 4-3-2-2.
2. The "HV" scale was set at 0.8
3. A quinine sulfate standard at 2.5 microgm./ml. was read at an excitation wavelength of 350mu and an emission wavelength of 450 mu at the above settings using a 30% scale. The fluorescence of this solution remained stable over long periods and was utilized to standardize the spectrofluorimeter for each experiment.

4. Tubes containing histamine with the appropriate standards were read at wavelengths of 350mu and 450mu. Spermidine were read at 350mu and 400mu.

SENSITIVITY OF THE METHOD

To assure more complete separation and recovery of histamine and spermidine the columns were eluted with the appropriate solution until no trace of the amine in question could be detected in the eluate. This required volumes of eluent greater than those recommended in the original procedure of Michaelson and Coffman (1969). The recoveries after passing standard amounts of histamine or spermidine through the entire procedure, alone and in combination with tissue, are presented in Tables A and B.

STATISTICS

The paired "t"-test was employed in calculating levels of significance.

QUALITY OF RECOVERY OF HISTAMINE FROM THE METHOD OF MICHAELSON & COFFMAN (1969)

TISSUE*	AMT. ADDED (ng)	AMT. RECOV'D. (ng)	% RECOVERY
none	50	49.5,52.5	102
Whole brain	none	340	
Whole brain	50	387	94
none	100	98.7,96.1	97.4
Liver	none	226	
Liver	100	319	93
none	200	201.5,196	99.4
Whole brain	none	352	
Whole brain	200	535	91
none	300	292,296	98
Whole brain	none	331	
Whole brain	300	620	92

*All tissues collected at 1000 hr. from entrained animals

TABLE A

QUALITY OF RECOVERY OF SPERMIDINE FROM THE METHOD OF MICHAELSON & COFFMAN (1969)

TISSUE*	AMT. ADDED (ug)	AMT. RECOV'D. (ug)	% RECOVERY
none	25	22.8,25.0	96
Whole brain	none	117	
Whole brain	25	140	92
none	50	50.5,47.1	97
Liver	none	46	
Liver	50	92	92
none	100	95.5,98.7	97
none	150	148.5,146	98
Whole brain	none	106	
Whole brain	150	244	92

*All tissues collected at 1000 hr. from entrained mice

TABLE B

RESULTS

GENERAL PHARMACOLOGY

Acute intravenous toxicity

Injection of sub-lethal doses of spermidine intravenously resulted in a consistent sequence of physiologic changes. Either immediately after the injection or occasionally before it was even completed, animals entered a brief period lasting from fifteen to thirty seconds characterized by increased motor activity interrupted frequently by abrupt jumping high into the air. These signs invariably preceded all other signs of toxicity and in their absence no further signs appeared. Most fatalities occurred without progression to further stages and no deaths occurred that were not preceded by these signs. For purposes of future discussion, this period will be referred to as Stage I in the manifestations of spermidine toxicity. It was followed immediately by a period during which the animal remained rather stationary in a crouched position. Respirations became hyperpneic and labored with audible

gasping. Heart rate was regular and rapid. Vasodilatation was evident in the vessels of the ears and skin. Excessive salivation, lacrimation or defecation were not observed. Most affected animals were quite sensitive to the slightest tactile or auditory stimulus, jumping into the air when touched or with hand-clapping. The few animals that attempted to ambulate were clearly ataxic. This period, henceforth called Stage II, lasted up to fifteen minutes. All further fatalities occurred during this stage. Stage III was characterized by a gradual return of spontaneous motor activity and feeding behavior. Respiratory and heart rates declined to normal. Likewise, the piloerection and vasodilatation gradually disappeared. A more detailed evaluation of neurologic function was possible during this period. Animals were unable to balance on a glass rod at this time. Initial attempts at walking were characterized by dragging of the hind legs which were typically in a hyperextended posture with marked increase in motor tonus. Even after this resolved, their gait continued to be quite unsteady with weaving and frequent falls after which the animal had a great deal of difficulty righting itself. A mild hypersensitivity to tactile and auditory stimuli occasionally persisted into this stage. No fatalities occurred during Stage III. Diminishing signs of toxicity could be observed up to two hours after

injection in animals receiving doses approaching the LD 50. There was no observable difference between mice who survived even potentially lethal doses of spermidine and those who received injections of normal saline.

Post-mortem examination of fatalities revealed only non-specific gross pathologic changes. Examination of the heart revealed a deeply cyanotic myocardium implying significant hypoxia and hypercarbia. Excised lungs floated well in water, indicating the absence of a major accumulation of pulmonary edema. The remainder of the major organ systems showed no obvious changes.

The LD 50 of spermidine phosphate varied significantly during the imposed light-dark cycle as shown in Table 1 and, in graphic form, in Figure 3. Peak LD 50 (lowest toxicity) occurred near the onset of the dark phase of the cycle. The lowest LD 50 (peak toxicity) was recorded just before the onset of the daylight phase. The 54.3% difference between these two points is highly significant ($p < .0025$). No alteration in the usual signs of toxicity nor their sequence was seen during the twenty-four hour cycle despite the change in the LD 50. The slope of the equation describing the LD 50 function also varied significantly. The steepest slope was recorded at the time of peak toxicity while the opposite was true of the time of least toxicity.

THE DIURNAL VARIATION OF THE LD 50 OF INTRAVENOUS SPERMIDINE

TIME (EDT)	LD 50 (mg/kg) + 1 S.D.	SLOPE
0115	97 ± 5.7	25
0415	92 ± 2.0*	68
0815	112 ± 7.5	29
1215	108 ± 8.4	19
1615	115 ± 9.0	19
2015	142 ± 18 *	11

* $p < .0025$

n = 5

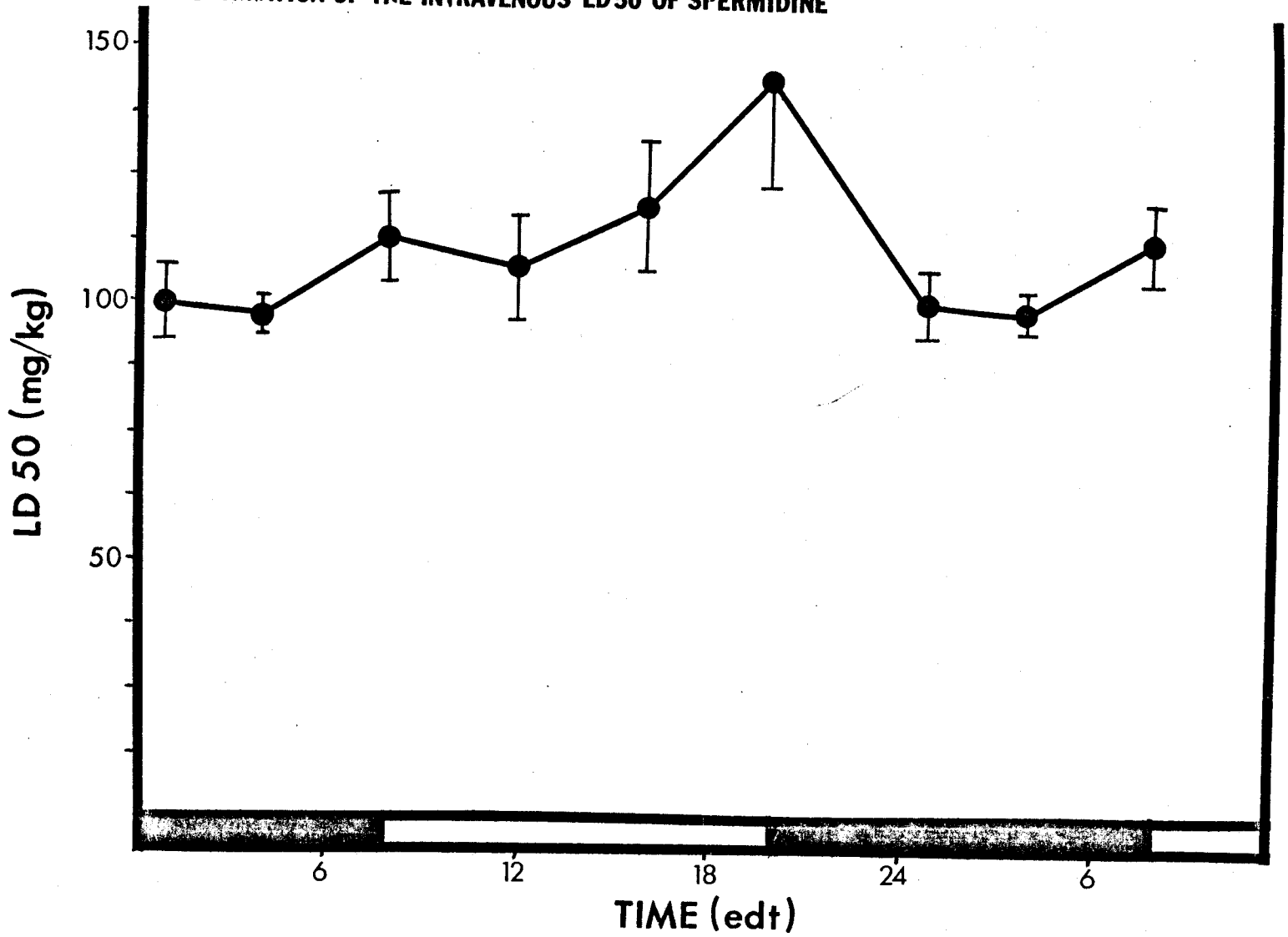
TABLE 1

Legend for figure 3 and subsequent similar figures:

abscissa = time in hours. Black bar represents period of darkness and open bar represents period of daylight.

vertical brackets = $\pm$ 1 S.D.

FIGURE 3. DIURNAL VARIATION OF THE INTRAVENOUS LD50 OF SPERMIDINE



Intraperitoneal Toxicity

The signs of toxicity after intraperitoneal injection were similar but not identical to those seen after intravenous injection. As expected, the signs of toxicity evolved in a more gradual fashion and persisted for a longer period of time. Without exception, the features of Stage I of the intravenous toxicity were not seen after intraperitoneal injection. Depending on the dose, the initial indications of toxicity were seen as soon as two to three minutes after injection, or they might be delayed as long as ten minutes. The first manifestations consisted of signs which corresponded in essence and sequence to those described as Stage II of intravenous toxicity. Thus, respiratory distress, tachycardia and vasodilatation appeared over a five to fifteen minute period. Hypersensitivity to environmental stimuli could be demonstrated soon thereafter. Animals who attempted to walk were clearly unsteady and had a greatly impaired ability to right themselves after their frequent falls. At lower dose levels, these signs might be quite transient but at doses approaching the LD 50 they often persisted as long as three to four hours. Subsequently all signs subsided excepting the ataxic gait which was not seen after approximately four and one half hours. Deaths did not seem related to any specific stage of the intraperitoneal

toxicity although very few animals died who survived the stage of respiratory distress. No fatalities occurred after four and one half hours. No observable differences could be found between treated and control animals after that time.

As with the intravenous toxicity, a significant diurnal variation in the intraperitoneal LD 50 was found (the peak LD 50 differed from the trough LD 50 by 52.1%). A rise in the LD 50 was recorded near the transition from light to dark as seen with intravenous doses. In addition, another more variable peak was recorded just before the onset of light. The intraperitoneal LD 50 remained four to five times the value found with the intravenous route. Again the slope of the LD 50 function was greatest during periods of peak toxicity.

THE DIURNAL VARIATION OF THE LD 50 OF INTRAPERITONEAL SPERMIDINE

TIME (EDT)	LD 50 (mg/kg) + 1 S.D.	SLOPE
0200	537 ± 126	2.89
0600	791 ± 241 *	3.60
1000	583 ± 56	11.37
1400	520 ± 46 *,**	12.30
1800	617 ± 61 **	2.81
2200	535 ± 66	8.85

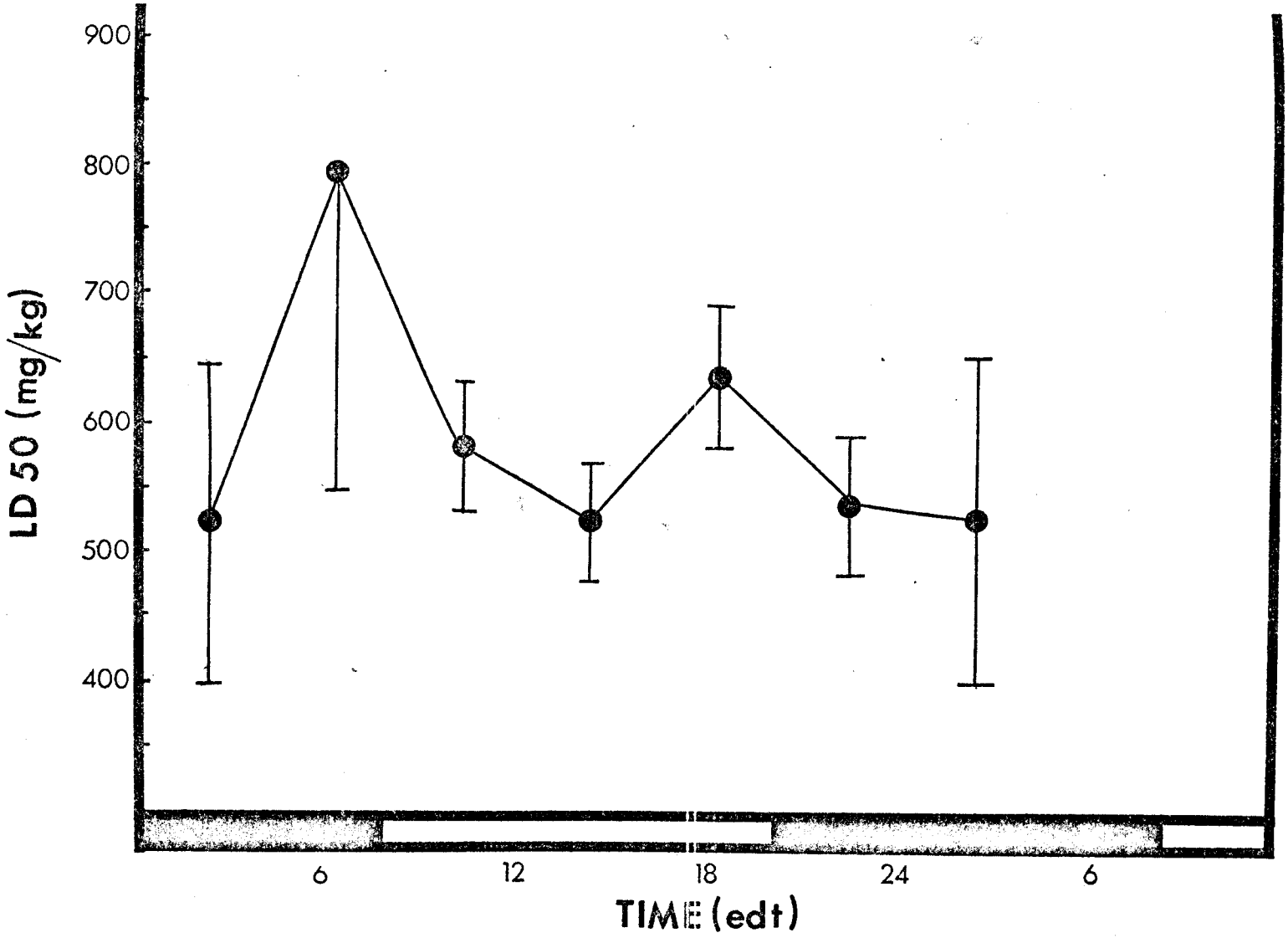
* $p < .025$

** $p < .02$

n = 9

TABLE 2

FIGURE 4. DIURNAL VARIATION OF THE INTRAPERITONEAL LD 50 OF SPERMIDINE



Protection from spermidine toxicity

As seen in table 3, tripelenamine tends to reduce the mortality from intraperitoneal doses of spermidine. However, this reached statistical significance only at a dose of 15 mg/kg of tripelenamine and the 500 mg/kg dose of spermidine. Higher doses of tripelenamine were found to produce signs of toxicity of themselves (lethargy, ataxia and occasional fatalities). This protective effect has been previously reported from this laboratory (Friedman and Rodichok, 1970).

Cyproheptadine was considerably more effective in protecting from spermidine toxicity as seen in Table 4. At a dose of 0.5 mg/kg there was a highly significant increase in the LD 50 (reduced toxicity) as compared to unprotected animals ($p < .02$). Fatalities in the protected group showed the usual signs of spermidine toxicity.

MORTALITY AFTER INTRAPERITONEAL SPERMIDINE.

PROTECTION WITH TRIPELENAMINE.

	PRE-TREATMENT			
	NONE	TRIPELENAMINE		
DOSE OF SPD		5.0 mg/kg	10 mg/kg	15 mg/kg
1000 mg/kg	11/11	11/11	11/11	11/11
750 mg/kg	9/11	10/11	6/11	5/11
500 mg/kg	6/11	6/11	5/11	2/11
LD 50 (mg/kg) ± 1 S.D.	468± * 125			792 ± * 123

* p = .05

n = 11

TABLE 3

MORTALITY AFTER INTRAPERITONEAL SPERMIDINE.

PROTECTION WITH CYPROHEPTADINE

	PRE-TREATMENT			
	NONE	CYPROHEPTADINE		
DOSE OF SPD		0.1 mg/kg	0.25 mg/kg	0.5 mg/kg
1000 mg/kg	11/11	11/11	11/11	9/11
750 mg/kg	9/11	9/11	6/11	2/11
500 mg/kg	6/11			
LD 50 (mg/kg) ± 1 S.D.	468 ± * 125			865 ± * 107

* $p < .02$

n = 11

TABLE 4

MOTOR ACTIVITY RHYTHM IN MICE

The true circadian nature of motor activity rhythms in rodents is well known (DeCoursey, 1960). As seen in Figure 5, this rhythm was well entrained by the environmental conditions used in this study. Peak activity occurred in anticipation of the onset of darkness and gradually receded as daylight approached. An inconsistent peak often occurs at the onset of light as in this study. The difference between peak and trough values is significant ($p < .001$). The secondary peak at the onset of daylight did not reach statistical significance in this study.

Figure 6 depects in graphic form the correlation between the motor activity rhythm and the rhythm for intravenous spermidine toxicity. Both peak LD 50 and peak motor activity just anticipate the onset of darkness. Similarly, a less significant peak can be seen in both rhythms at the onset of daylight.

The correspondence between the rhythm of intraperitoneal spermidine toxicity and motor activity is less remarkable as seen in Figure 7. Nevertheless, significant peaks occur in the toxicity rhythm at approximately the same times as in the activity rhythm.

Figure 5. Diurnal variation of the motor activity of mice

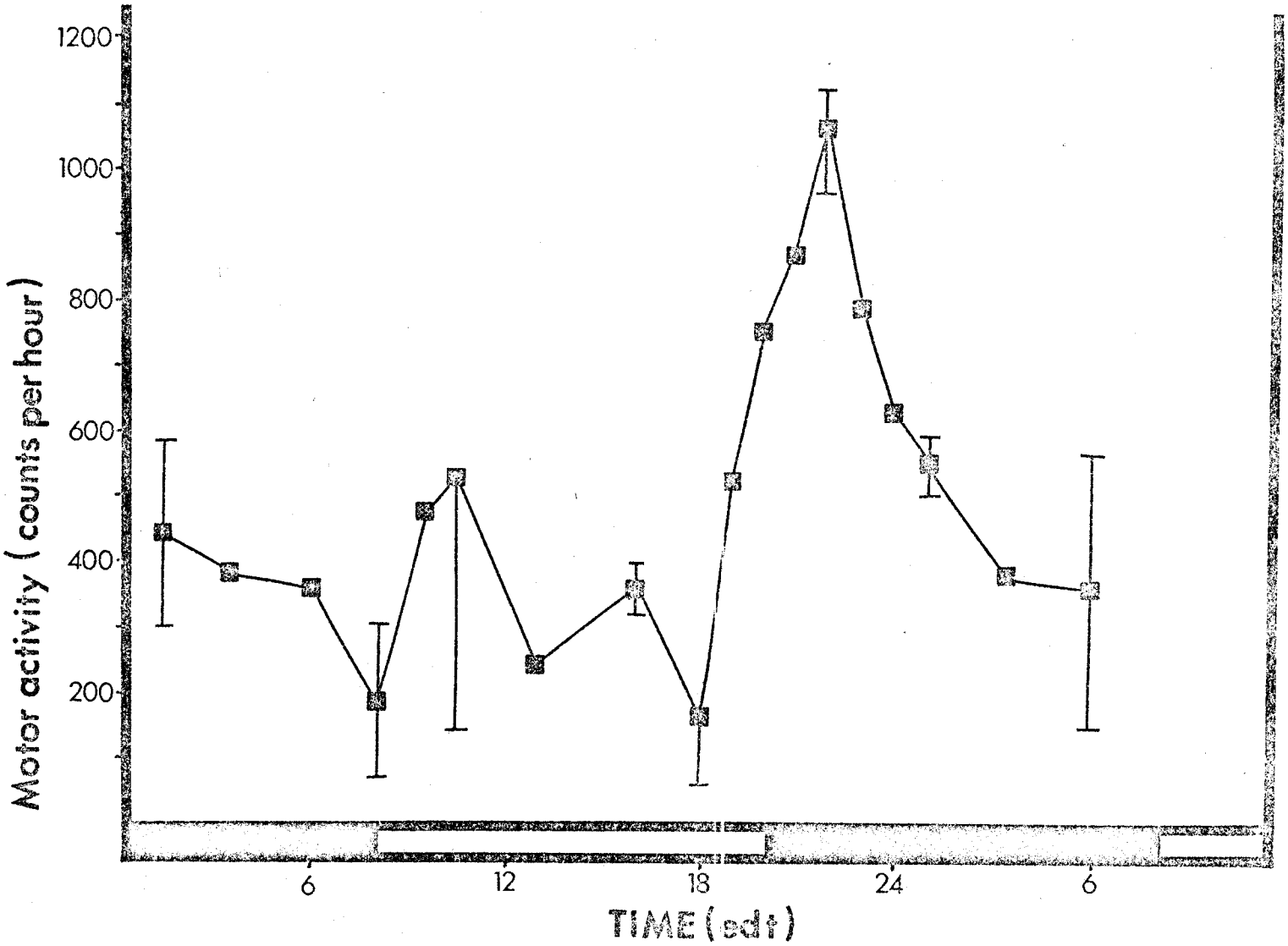


FIGURE 6. DIURNAL VARIATION OF THE INTRAVENOUS TOXICITY OF SPERMIDINE

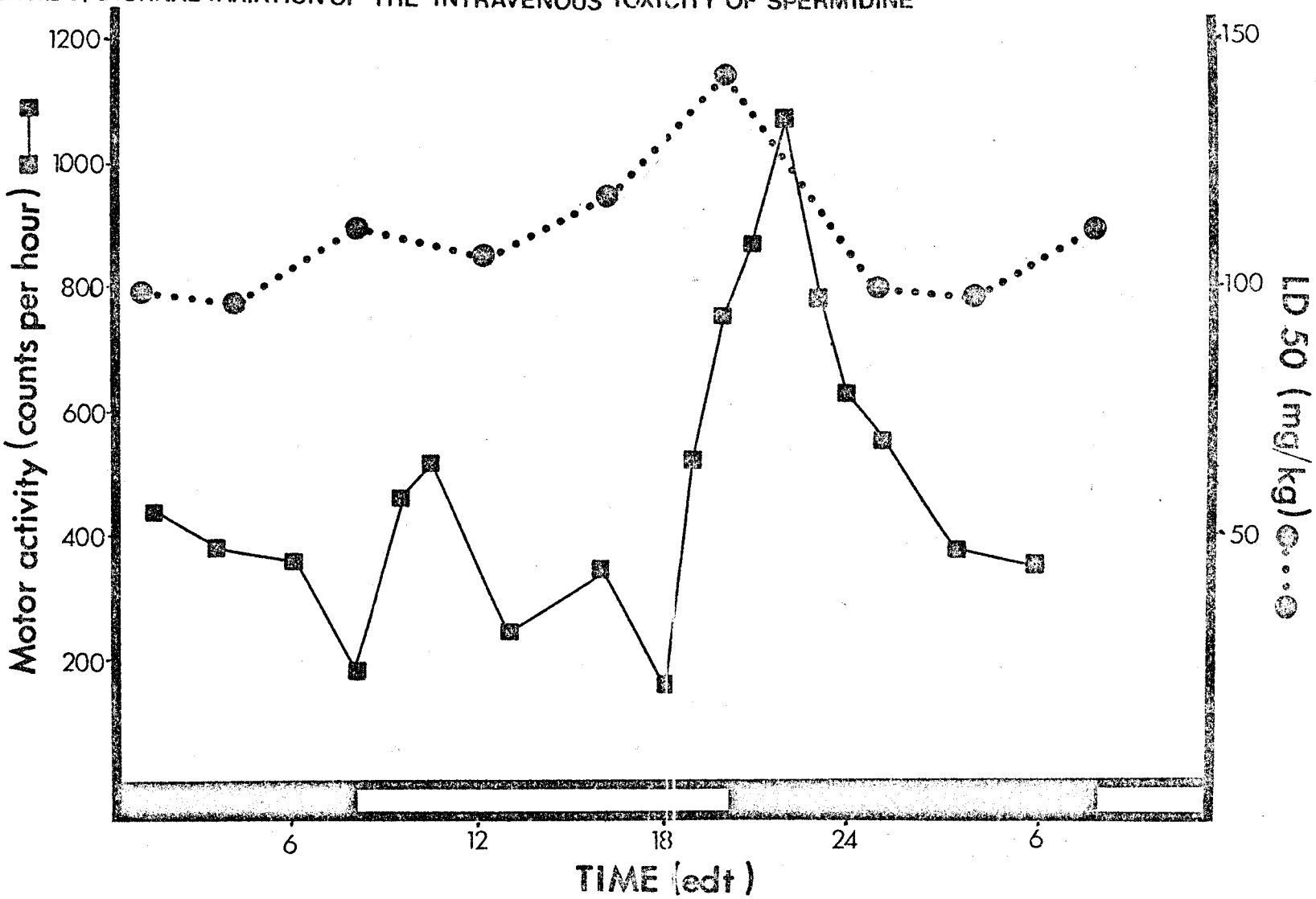
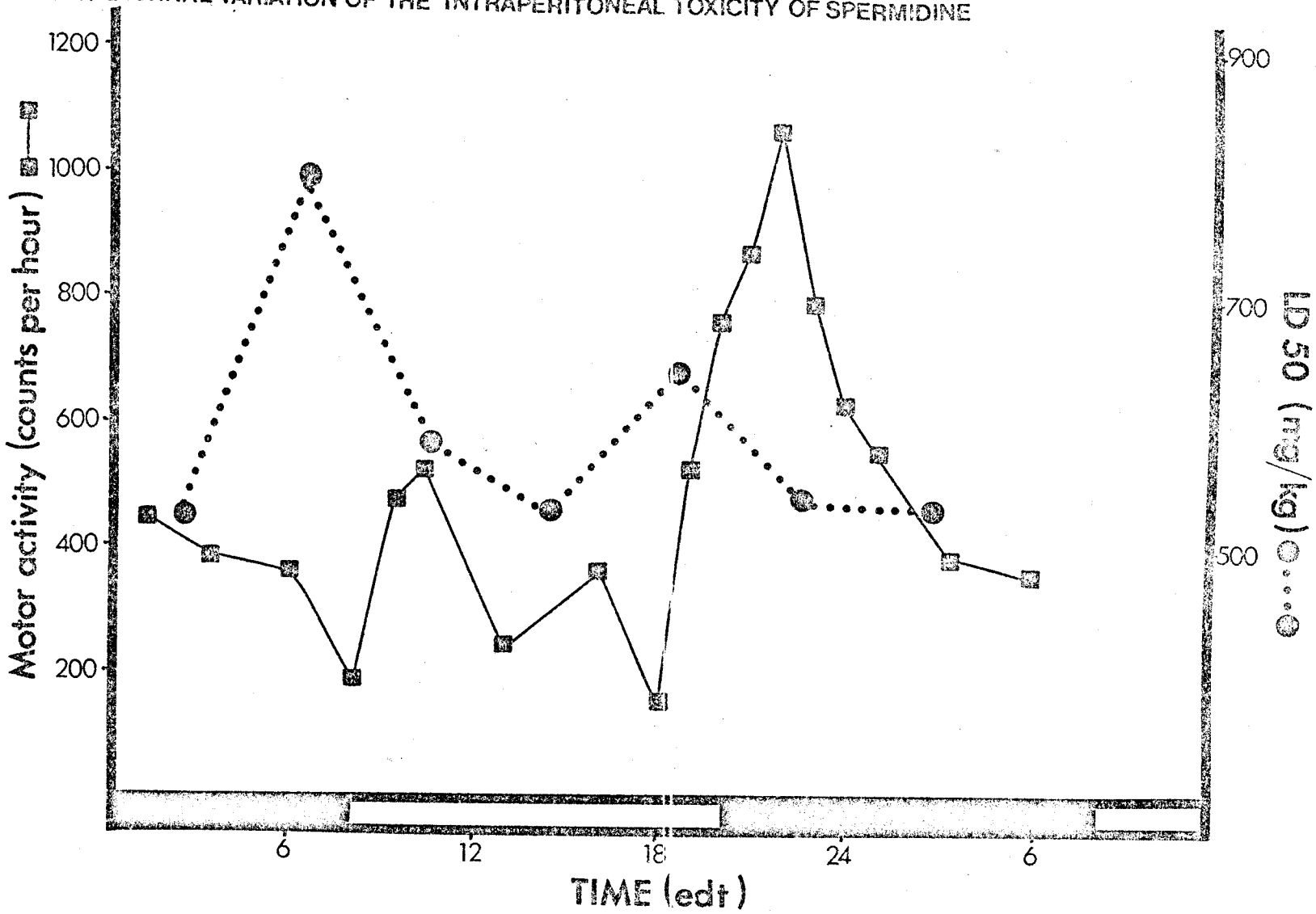


FIGURE 7. DIURNAL VARIATION OF THE INTRAPERITONEAL TOXICITY OF SPERMIDINE

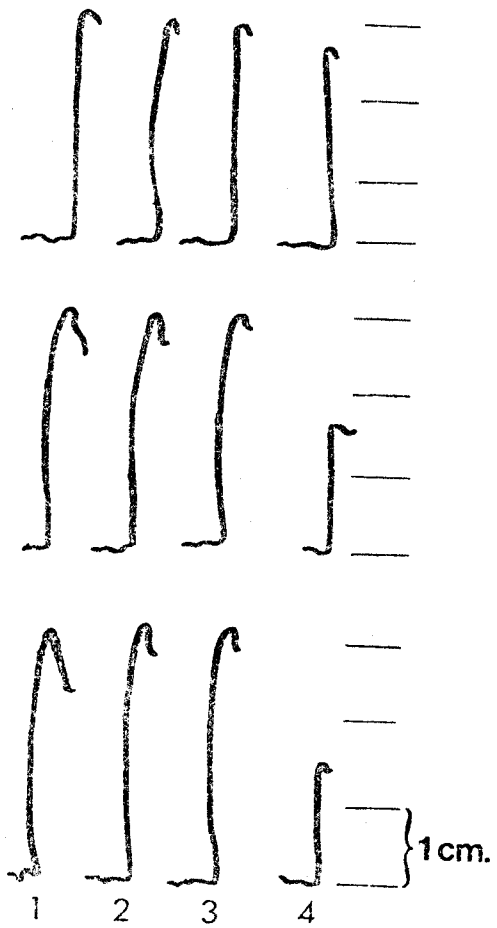


IN VITRO ACTIVITY

Spermidine had no effect on the acetylcholine-induced contraction of guinea-pig ileum when injected after the agonist. A consistent reduction in the amplitude of contractile response was seen however when spermidine was injected ten seconds prior to the acetylcholine. At a final concentration of 0.8uM spermidine resulted in approximately a 20% reduction in amplitude. At concentrations of 1.6uM and 3.2uM, a 50% reduction was seen. Higher concentrations produced no greater effect. Control injections of Tyrode's solution had no effect. When injected more than ten seconds prior to the agonist, spermidine had no effect. Spermidine did not affect contractions induced with barium. Figure 8 shows representative examples of the effect of spermidine injected ten seconds before acetylcholine.

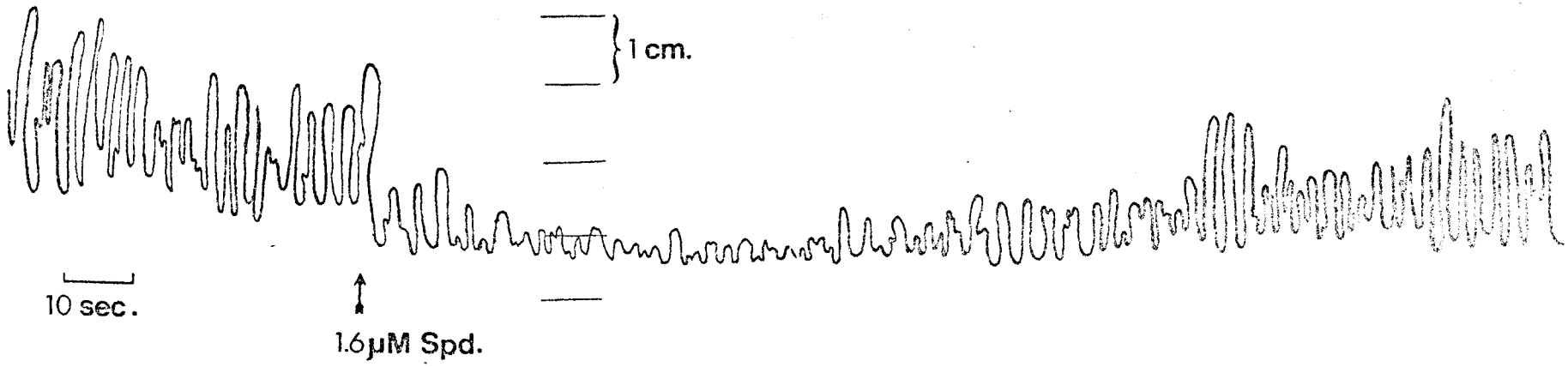
At increased sensitivity (.02mv/cm) the effect of spermidine on the spontaneous activity of the guinea-pig ileum can be seen (Figure 9). At a final concentration of 1.6uM there is a significant reduction of the amplitude of the spontaneous contractions although the frequency is unchanged. A small depression of the baseline muscle tone occurs. Control injections of Tyrode's solution had no effect.

Figure 8. The effect of spermidine on the acetylcholine-induced contraction of guinea pig ileum



Responses 1-3 represent consecutive control injections of Ach (4.5μg/ml). The fourth was preceded (10 sec.) by an injection of spermidine at the concentration indicated.

Figure 9. The effect of spermidine on the spontaneous activity of guinea pig ileum

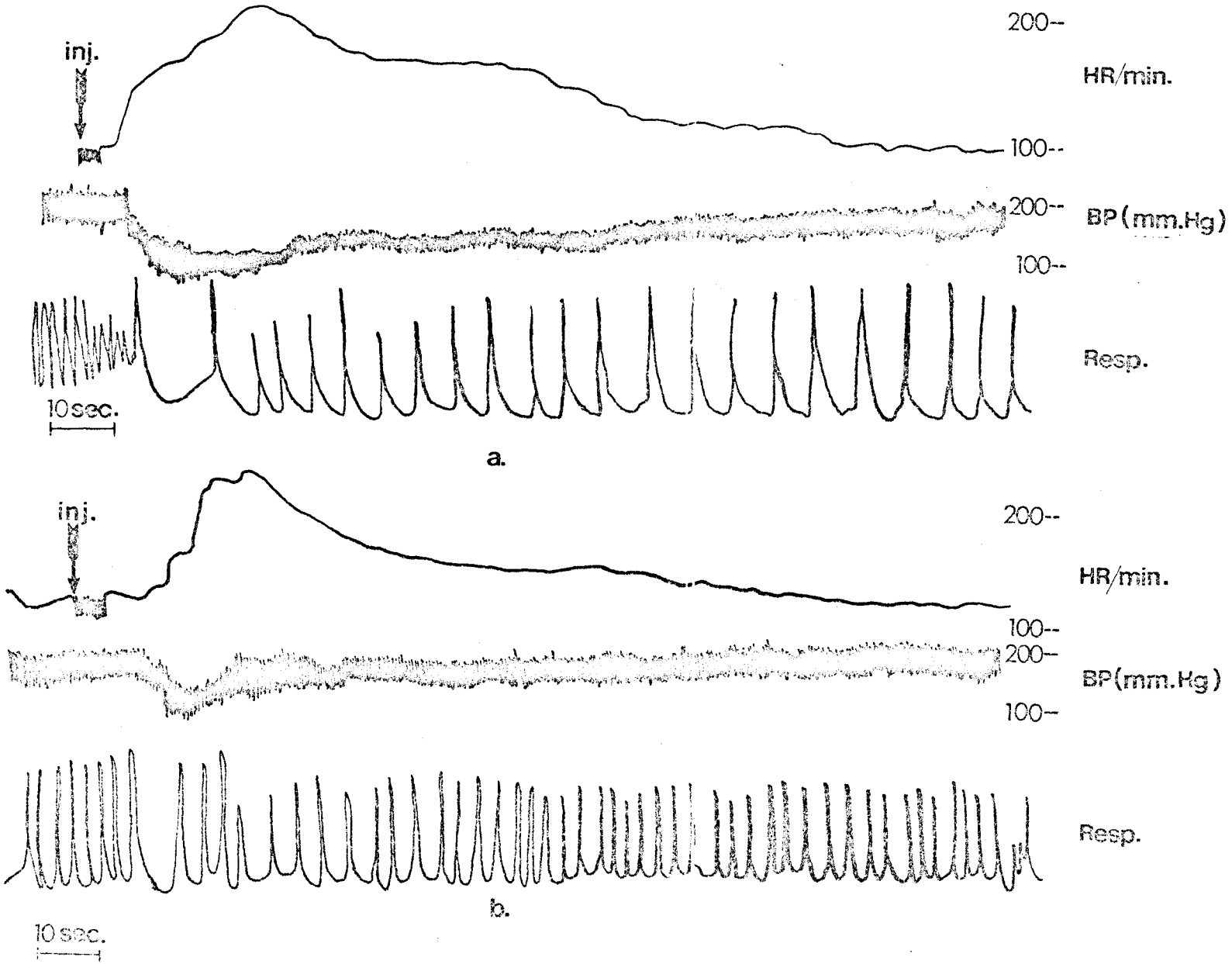


IN VIVO EFFECTS OF SPERMIDINE IN DOGS

Injection of 0.75 - 1.00 mg/kg dose of spermidine intravenously was sufficient to produce a marked cardio-pulmonary response. A prompt reduction in the mean blood pressure and a rise in the pulse pressure were associated with hyperpnea and tachycardia. These parameters showed a gradual recovery over the following one to two minutes (Figure 10). Injections of histamine at a dose of 5 ug/kg produced a very similar response (Figure 10). Following the first effective dose of spermidine, subsequent injections were of little or no significant effect.

Intravenous injection of 200 mg/kg spermidine caused instantaneous loss of detectable blood pressure and irreversible apnea. Cardiac electrical activity was detectable for a short time thereafter, showing marked ST-T segment depression and T-wave inversion.

FIGURE 10. RESPONSE TO INTRAVENOUS SPERMIDINE (a) & HISTAMINE (b) IN THE DOG



BARBITURATE-INDUCED SLEEPING TIME IN MICE

1. The effect of pre-treatment with spermidine on the duration of barbiturate-induced sleeping time.

   Pre-treatment with doses of spermidine significantly prolonged sleep after sodium pentobarbital. This effect was clearly maximal when the spermidine was given thirty minutes prior to sleep induction. A suggestion of a similar result was seen with doses given sixty minutes before while no change occurred with pre-treatment at 120 minutes. A dose of 300 mg/kg was approximately twice as effective as 150 mg/kg. It is of interest to note the mild alteration in duration produced by sham pre-treatment with physiologic saline. Though not statistically significant, all subsequent studies utilize this form of control for reasons to be discussed later. Table 5 summarizes these data.

2. Diurnal variability of the duration of barbiturate-induced sleeping time and the effect of pre-treatment with spermidine thereon.

   The duration of barbiturate-induced sleep in mice varied significantly during the programmed twenty-four hour light-dark cycle (Table 6 and Figure 11). Peak duration occurred in anticipation of the onset of daylight while a trough was seen

just prior to the onset of the dark phase. The difference between these values is significant ($p < .001$). As seen in Figure 11, the duration of sleep was inversely related to motor activity.

The prolongation of sleep produced by pre-treatment with spermidine was likewise time-dependent (Table 6). The diurnal curve maintains the same phase relationships as with barbiturate alone (Figure 11), peak sleeping time occurring just prior to the onset of daylight. Spermidine seemed to abolish the trough appearing just prior to the onset of darkness, the point at which the greatest relative increase over control was seen (Table 6). This is the time period of least toxicity of spermidine.

THE EFFECT OF PRETREATMENT WITH SPERMIDINE ON BARBITURATE-INDUCED SLEEP

Minutes prior to barbiturate inj.	DURATION OF SLEEP (Minutes ± 1 S.D.) Pre-Treatment			
	none	saline	Spd-150mg/kg	Spd-300mg/kg
30	41± 3	45± 6	57± 3*	71± 2**
60	41± 3	45± 6	48± 2	53± 3
120	41± 3	45± 6	38± 3	41± 2

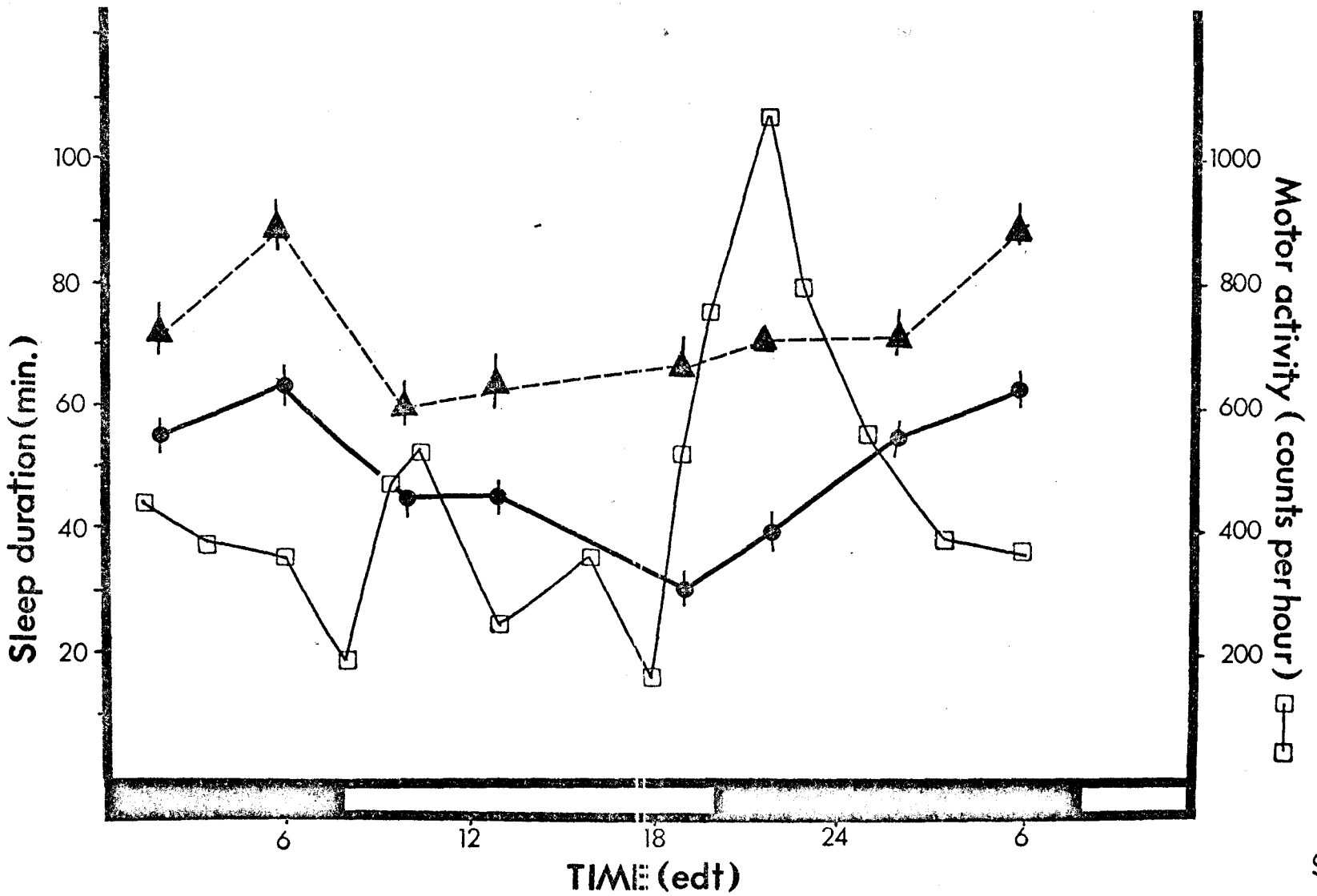
* $p < .05$ (versus saline control)

** $p < .001$ (versus saline control)

n = 5

TABLE 5

FIGURE 11. DIURNAL VARIATION OF SLEEP AFTER PENTOBARBITAL (●—●) INCREASED BY SPERMIDINE (▲--▲)



DIURNAL VARIABILITY IN THE DURATION OF SLEEP AFTER PENTOBARBITAL ALTERED BY PRETREATMENT WITH SPERMIDINE

	Duration of sleep (minutes ± 1 S.D.) Pre-Treatment		% increase
Time	saline	Spd-300mg/kg	
0200	54± 4.6	71± 2.6	32 (p < .01)
0600	63± 7.5 **	76± 7.2***	38 (p < .05)
1000	44± 5.2*	60± 2.2***	36 (p < .02)
1300	43± 2.9	62± 6.7	45 (p < .05)
1900	29± 3.0**	67± 4.9	135(p <.001)
2200	39± 5.4	71± 1.0	86 (p <.001)

* p <.05

** p <.001

*** p <.001

n= 5

TABLE 6

3. Interaction between central biogenic amine depletion, sleeping time and spermidine.

Decaborane has been shown to deplete the levels of several biogenic amines in the CNS, maximally at a point twenty-four hours after injection (von Euler and Lishajko, 1965; Merritt and Sulkowski, 1967; Medina et al., 1969). No attempt was made to duplicate all of these studies. The effect of decaborane on central histamine levels was confirmed. In addition, a significant reduction of central spermidine was demonstrated as seen in Table 7.

Pre-treatment with decaborane markedly prolonged the duration of sleep after pentobarbital. Whereas a dose of pentobarbital of 30 mg/kg. is insufficient to produce sleep in control animals. A dose of 60 mg/kg was followed by only 42 ± 3 minutes of sleep in the control group. At this dose of pentobarbital, of twelve animals pre-treated with decaborane six died after periods of deep sleep and six slept for periods exceeding 120 minutes but survived. Spermidine alone did not produce sleep in pre-treated animals nor did the usual signs of toxicity seem altered. Pretreatment with both decaborane and spermidine significantly prolonged the duration of sleep as compared to pre-treatment with spermidine alone but this combination was less effective than decaborane alone. These data are summarized in Table 8.

REDUCTION OF WHOLE BRAIN HISTAMINE AND SPERMIDINE LEVELS

TWENTY-FOUR HOURS AFTER DECABORANE

	Whole brain histamine (ng/gm ± 1 S.D.)	Whole brain spermidine (ug/gm ± 1 S.D.)
Control	318 ± 9*	109 ± 7**
Decaborane	168 ± 12*	82 ± 8**

* $p < .001$

** $p < .05$

n = 3

TABLE 7

THE EFFECT OF PRETREATMENT WITH DECABORANE &/or SPERMIDINE

ON THE DURATION OF SLEEP AFTER PENTOBARBITAL

Pretreatment		Pentobarbital		sleep duration
Db	Spd	30mg/kg	60mg/kg	(min.± 1 S.D.)
-	-	+	-	0
-	-	-	+	42 ± 3
-	+	+	-	15 ± 2*
-	+	-	+	60 ± 2
+	-	+	-	70 ± 5*
+	-	-	+	6 deaths & 6 slept 120 min.
+	+	-	-	0
+	+	+	-	41 ± 4
+	+	-	+	12/12 deaths

Db = Decaborane, 15mg/kg intraperitoneally 24 hours prior to sleep-induction

Spd = Spermidine, 300 mg/kg i.p. 30 min. prior to sleep-induction

* $p < .001$ (versus control injection of corresponding dose of pentobarbital alone)

TABLE 8

4. The time course of histamine and spermidine levels during sleep induced by pentobarbital.

Table 9 demonstrates the reduction in central histamine levels during barbiturate-induced sleep. A maximum reduction of 22% was seen forty-five minutes after the onset of sleep. Significant reduction of midbrain and caudate histamine levels by pentobarbital has been reported previously in rats (Friedman, A.H. and Walker,C.A.,1969). Little change occurred in the levels of spermidine during sleep , the maximum reduction being 11% at forty-five minutes (Table 9).

DIURNAL VARIATION OF HISTAMINE AND SPERMIDINE LEVELS IN WHOLE BRAIN,MYELENCEPHALON AND LIVER.

HISTAMINE

Levels of histamine were highest in "whole brain"(mesencephalon, hypothalamus-thalamus and hemispheres). Significant levels were also detected in the medulla-pons and liver. During the programmed light-dark cycle peak levels occurred in all three tissues just prior to the onset of the dark phase. Lowest levels were reached within four hours after the onset of darkness (Table 10 and Figure 12).

TIME COURSE OF WHOLE BRAIN HISTAMINE AND SPERMIDINE

DURING BARBITURATE-INDUCED SLEEP

	minutes after injection					
	control	15	30	45	60	control
Whole brain histamine (ng / gm)	* 222	202	197	* 173	202	225
Whole brain spermidine (ug / gm)	45.5	48.5	42.5	40.0	43.0	48.0

n = 2

*p = .01 - four control animals (226 ± 6.1 ng/gm) compared to four animals collected at 30 & 45 minutes (185 ± 14 ng/gm)

TABLE 9

SPERMIDINE

Levels of spermidine were higher in the medulla-pons than in the whole brain. Relatively low concentrations were detected in the liver. Levels in all three tissues showed a diurnal variability similar to that of histamine. Peaks occurred just before the onset of darkness while lowest levels were found at the onset of the day-light phase. In the case of the medulla-pons and whole brain , higher levels were still present four hours after the onset of darkness (Table 10 and Figure 13).

DIURNAL VARIABILITY IN THE LEVELS OF HISTAMINE AND SPERMIDINE IN WHOLE BRAIN, MYELENCEPHALON & LIVER

		Time (EDT)			
		0800	1300	1800	2300
Histamine (ng/gm ± 1 S.D.)	Whole brain	210 ± 20	242 ± 11	350 ± 26 *	186 ± 4 *
	Myelencephalon	136 ± 9	148 ± 4	201 ± 76 **	165 ± 58 **
	Liver	90 ± 31	186 ± 27	221 ± 4 *	93 ± 18 *
	* p < .001 ** NS n = 4				
Spermidine (ug/gm ± 1 S.D.)	Whole brain	47 ± 4 *	57 ± 9	110 ± 6 *	82 ± 7
	Myelencephalon	80 ± 8 *	92 ± 10	155 ± 15 *	155 ± 50
	Liver	16 ± 3 *	26 ± 7	49 ± 3 *	18 ± 2
	* p < .001 n = 4				

TABLE 10

FIGURE 12. DIURNAL VARIABILITY OF HISTAMINE LEVELS

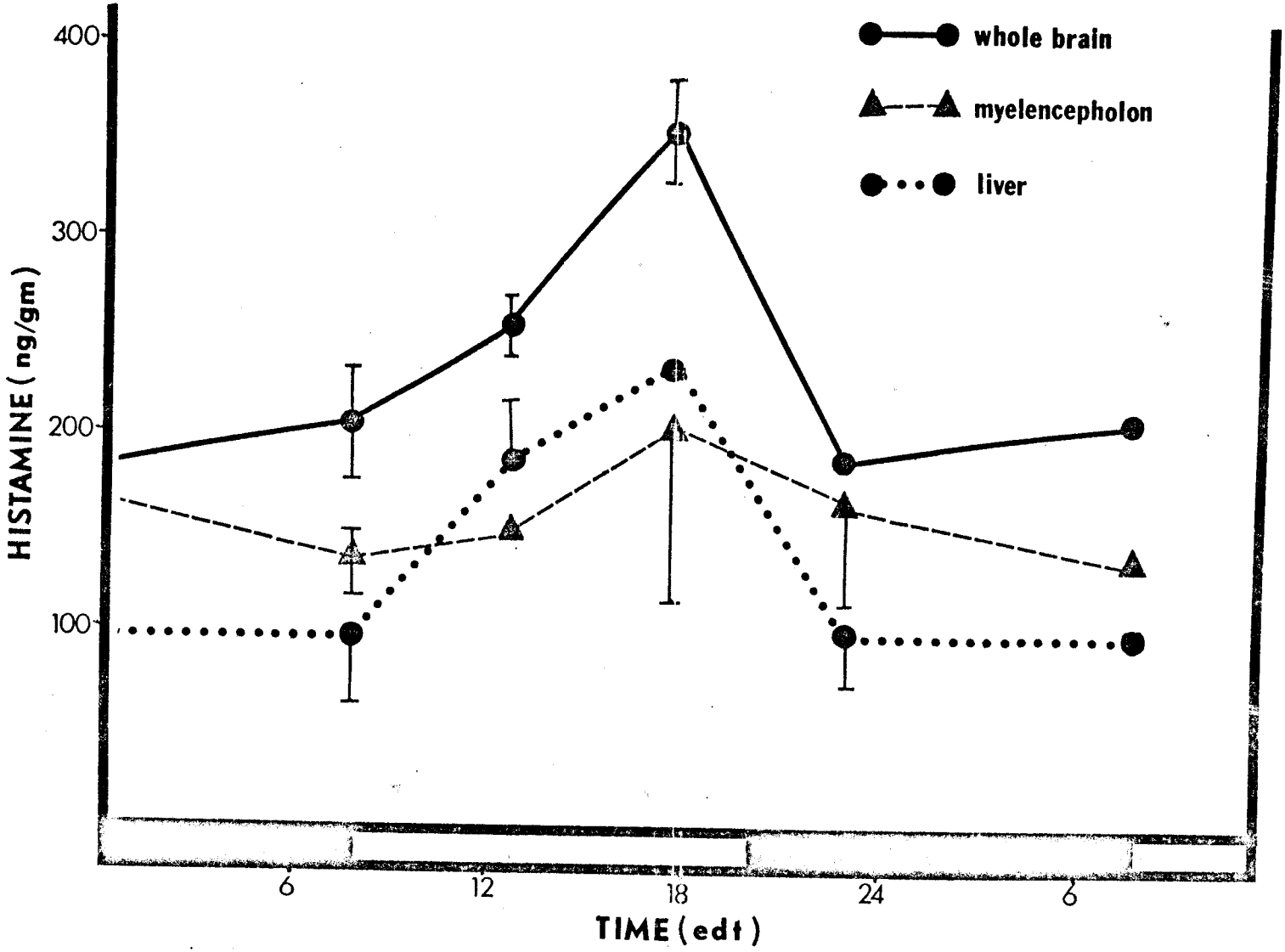
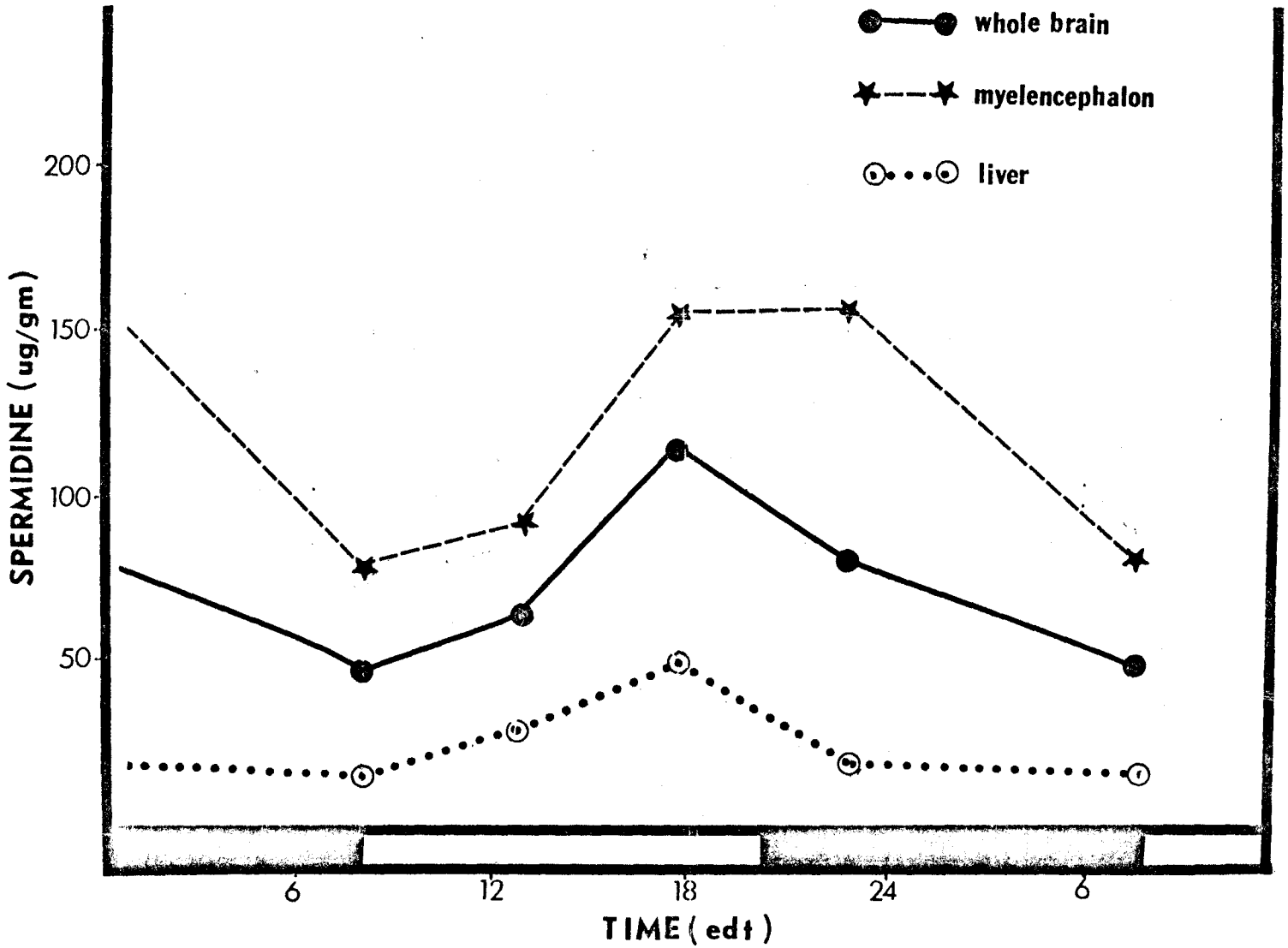


FIGURE 13. DIURNAL VARIABILITY OF SPERMIDINE LEVELS



DISCUSSION

Few studies of the *in vivo* acute toxicity of spermidine have been reported. Intravenous injections of both spermine and spermidine have been reported to cause transient hypotension and respiratory distress (Tabor, C.W., and Rosenthal, S.M., 1956). In a more recent study, Shaw (1972) reported sedation, respiratory distress and ataxia after parenteral injections of both spermine and spermidine. Lethal doses were attended by clonic convulsions as well. In the present study intravenous injections of spermidine in mice were followed immediately by a brief period of hyperactivity (Stage I). Stage II of the intravenous toxicity and the first signs of intraperitoneal toxicity were marked by severe respiratory distress, tachycardia and cutaneous vasodilatation. Deaths occurred in either Stage I (intravenous) or Stage II and were often preceded by agonal myoclonic seizure activity. Survivors of these stages showed a variable period (Stage III) of ataxia, motor retardation and hypersensitivity to noise and tactile stimuli. In sub-lethal doses little effect was seen on rectal temperature. Although a direct action of spermidine cannot be excluded, these effects are quite similar to those seen after injections of histamine (Douglas, W.W., 1968; Kahlson, G. and Rosengren, E., 1965). This is supported by the similarity in response to intravenous injection of histamine and spermidine in dogs

demonstrated here. Although this response was easily distinguished from that of serotonin in the dog, the effects of this amine vary with different species and are often quite similar to those of histamine. Cyproheptadine has been shown to have both potent anti-histaminic and anti-serotonergic properties (Stone, C.A. et al, 1961; Van Reizen, H., 1972). The fact that this compound offered greater protection from the toxicity of spermidine than did tripelenamine, an anti-histamine which has no significant anti-serotonergic activity, suggests that both histamine and serotonin may be released by spermidine. Neither spermidine nor spermine pass the blood brain barrier (Snyder, S.H. et al., 1973) and thus a primary central site of action can be dismissed. Platelets and masts cells, storage sites for both histamine and serotonin, are possible sites of action for spermidine. Other polyamines such as the polymer "48/80" are potent releasers of biogenic amines from these sources (Rothschild, A.M., 1970). In the present study, dogs showed a marked tachyphylaxis to spermidine injections. This could very well be due to depletion of histamine and serotonin stores after the first effective doses and would therefore lend support to the notion that spermidine acts by releasing these amines. Tachyphylaxis is characteristic of the pharmacology of both histamine and serotonin.

Effective and lethal doses of spermidine were considerably higher with intraperitoneal as compared to intravenous administration.

This might be explained on the basis of incomplete and/or slow absorption through the peritoneum, resulting in lower and less effective blood levels. An additional factor however might be that spermidine is inactivated during absorption by this route. Diamine oxidase catalyses the oxidation of both spermidine and histamine and is present in significant quantities along the gastrointestinal tract (Kim, K.S., et al., 1969). This enzyme may have oxidized a significant proportion of intraperitoneal doses of spermidine. Diurnal variation in the activity of this enzyme has not been studied and,if significant, could further complicate studies using this route.

Diurnal variability in the LD50 of spermidine has not been studied previously and must be considered in future experimentation. The intravenous LD50 in mice reported by Shaw (1972) (78 mg/kg) is somewhat lower than values reported here but a lighting regimen was not utilized. Factors underlying such a variation are likely to be multiple. The significant fluctuation in spermidine levels shown here may in part reflect changes in the activity of catabolic enzymes. The abrupt change in the slope of the intravenous LD50 curve seen at the point of highest toxicity is significant. Steepening of the curve must indicate either an alteration of the turnover of spermidine or heightened receptor sensitivity (Nelson, W., and Halberg, F., 1973). The correlation of lowest toxicity with peak endogenous levels of spermidine may point to a decrease in catabolism. The decrease in

spermidine toxicity occurs at a time when endogenous histamine levels are highest and serotonin levels are low. Endogenous levels of these as well as other biogenic amines often correlate with increased release and catabolism (Kahlson, C. and Rosengren, E., 1968; Héry, F. et al., 1972; Mandell, A.J. et al., 1974). The functional significance of such a correlation is therefore unclear without further study. Since no change in the manifestations of toxicity was seen within the twenty-four hour cycle, a change in mode of action is unlikely.

The in vitro activity of spermidine was not extensively studied here. Polyamines have been previously shown to antagonize the contraction of guinea pig ileum induced by a variety of agents including acetylcholine, histamine, 5-HT and nicotine (DeMeis, L., 1967; Onodera, K., et al, 1968). DeMeis and DePaula (1967) and Nagai et al.(1969) have both reported a specific inhibition of magnesium-activated ATP-ase of myofibrils, myosin B and native actomyosin by polyamines. A similar action on vascular smooth muscle might explain the hypotension and cutaneous vasodilatation seen after spermidine injection but would not account for many of the other manifestations.

The basis for the well-established hypnotic effect of barbiturates is not known. The duration of sleep after appropriate doses is dependent on many factors. Reduction of ambient room temperature below 30°C prolongs the duration of barbiturate-induced sleep (Raventos, J., 1938). Several drugs known to potentiate the hypnotic

effect of barbiturates owe their action to their ability to induce hypcthermia (Lessin, A.W., and Parkes, M.W., 1957). The environmental temperature was therefore kept constant during this study. Although lethal doses of spermidine were associated with agonal hypothermia, no significant change in rectal temperature was seen with doses used to evaluate the effect of spermidine on pentobarbital-induced sleeping time. At the dosage utilized in the present study (60 mg/kg) pentobarbital-induced sleep was attended by a decline of rectal temperature of approximately 5^{o}C. This was unaffected by pre-treatment with spermidine. Nevertheless, pretreatment with spermidine prolonged the duration of sleep after pentobarbital. Since spermidine does not pass the blood-brain barrier, the basis for this effect must be peripheral. This would not exclude the possibility of the release of a centrally active compound. Both serotonin and histamine have been shown to prolong drug-induced sleeping time and, as noted above, could have been released by spermidine (Fastier, F.N., et al., 1957). Polyamines might also conceivably inhibit the degradation of barbiturates by the liver, thus elevating CNS concentrations of the drug and prolonging sleeping time. As noted above,this would be consistent with the association of polyamines with active synthesis, particularly in the liver.

A diurnal rhythm in the duration of barbiturate-induced sleep as shown in the present study, has been reported previously (Davis, W.M., 1962; Lindsay H.A. and Kullman, V.S., 1966; Scheving, L.E. et al, 1968b).

In most nocturnal species peak duration occurs during darkness and corresponds to the period of peak motor activity and highest body temperatures. This may in part be related to the diurnal rhythm of hepatic drug metabolism (Radzialowski, F.M. and Bousquet, W.F., 1968; Jori, A., et al., 1971). However, recent studies have implicated a change in receptor sensitivity to barbiturates as well (Nelson, W. and Halberg, F., 1973).

Decaborane reduces central levels of norephinephrine and serotonin as well as histamine. (Von Euler, U.S. and Lishajko, F., 1965; Merritt, J.H. and Sulkowski, T.S., 1967; Schayer, R.W. and Reilly, M.A., 1971). Its effect on PST seen in this study certainly suggests that the effect of barbiturates on sleep involves one or more of these biogenic amines. In contrast to the rat (Friedman, A.H. and Walker, C.A., 1969) pentobarbital caused a small but significant reduction in central histamine levels, perhaps the basis of its synergism with decaborane. Even if spermidine releases peripheral stores of histamine or serotonin neither of these biogenic amines enter the CNS in effective concentrations. However, spermidine alone also prolonged PST. This effect was significantly increased during the transition from light to dark. This is the period of least toxicity of spermidine, suggesting that the action on PST is mediated differently, perhaps by competition for enzymes in the liver metabolizing the pentobarbital.

Daily fluctuation in the levels of many of the biogenic amines is well established (Scheving, L.A. et al, 1968a; Friedman, A.H. and Walker, C.A., 1969; Piepho, R.W. and Friedman, A.H., 1971; Saita, Y., 1971; Walker, C.A. et al., 1971). Peak levels of histamine in rat brain have been found early in the dark phase of the cycle (Friedman, A.H., and Walker, C.A., 1969; Garbarg, M. et al., 1974). In the present study of the mouse, peak levels of histamine in brain and liver just anticipated the dark phase. The association of elevation of histamine levels with the period of greatest motor activity is in keeping with recent interest in the activating effect of histamine on the CNS (Monnier, M. et al., 1970; Taylor, K.M. and Snyder, S.H., 1971). Depletion of histamine levels by decaborane correlated with increased sleeping time. Stress caused by cold or restraint is associated with increased turnover of histamine (Taylor, K.M. and Snyder, S.H., 1972). Intraventricular injections of histamine have a definite awakening effect (Monnier, M. et al., 1970).

Significant diurnal variability in polyamine levels has not been reported previously. The phase of this rhythm was the same as that of histamine. Recent studies have renewed interest in a possible interaction between histamine and spermidine. As discussed above,

both are associated with periods of rapid growth, particularly during embryogenesis. During these periods, a significant proportion of histamine is localized within the nucleus as is spermidine (Kuhar, M. et al., 1971; Young, A.B. et al., 1971; Snyder, S.H., and Kuhar, M., 1972). The parallel reduction of histamine and spermidine levels during barbiturate-induced sleep shown here supports this association. On the other hand, a significant fraction of histamine in adult neural tissue is associated with synaptic elements and has some features of a true neurotransmitter (Snyder, S.H. and Kuhar, M., 1972). Polyamines are not known to be associated with this fraction.

The studies reported here are clearly preliminary and incomplete. In studying the acute toxicity of spermidine a picture consistent with a release of histamine and serotonin was seen. The prolongation of barbiturate-induced sleeping time after pretreatment with spermidine may be mediated differently. The diurnal fluctuation in spermidine levels and toxicity has not been previously established and must be considered in future studies. Further investigation into the neuropharmacology of the polyamines seems warranted.

SUMMARY

Some pharmacologic properties of the polyamine spermidine have been studied. Toxicity in mice after intravenous injection consisted of acute respiratory distress and myoclonic seizures followed by a period of lethargy, ataxia and irritability. Intraperitoneal doses produced a similar sequence of signs evolving over a longer period. Significant diurnal variability of the LD 50 was found with a peak for both the i.v. and i.p. routes occurring during the transition from light to dark in a programmed 24 hour cycle of twelve hours of light and twelve hours of darkness. This peak corresponded to the peak of the daily motor activity rhythm. Pretreatment with either cyproheptadine or tripelenamine significantly reduced the LD 50 of spermidine. Intravenous doses of spermidine in dogs caused acute hypotension, tachycardia and tachypnea and were associated with marked tachyphylaxis. Injections of histamine produced a very similar picture. Neither the diurnal fluctuation of the LD 50 of spermidine nor its alteration by tripelenamine and cyproheptadine have been reported previously.

Pretreatment with spermidine reduced the amplitude of acetylcholine-induced contraction of guinea pig ileum. The amplitude of the intrinsic rhythmic activity was also transiently diminished after spermidine.

The diurnal variability in barbiturate-induced sleeping time was confirmed, peak duration occurring late in the dark phase of the cycle. Pretreatment with spermidine significantly prolonged sleeping time. This effect was variable in magnitude during the 24 hour cycle , peak prolongation occurring at the transition from light to dark , the time period of peak LD 50 of spermidine and highest levels of endogenous spermidine and histamine.

A diurnal rhythm of endogenous levels of spermdine and histamine was established in whole brain, myelencephalon and liver. Highest levels of both amines occurred just prior to the onset of darkness.

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